

IS COEVOLUTION GOING ANYWHERE?

AN INVESTIGATION OF SEX, VIRULENCE, AND RECIPROCAL ADAPTATION

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The overarching motivation of evolutionary ecology is to explain why natural populations harbor so much genetic and phenotypic diversity. This dissertation features two particularly important and puzzling forms of diversity: variation in reproductive mode and variation in species interactions. The ultimate conclusion of this dissertation is that host-parasite coevolution underlies variation in both.

Why do females produce genetically variable offspring, via sexual outcrossing?

The evolution and maintenance of sex remains one of the great enigmas of evolutionary biology. Chapter 1 lays the foundation for the problem. A combination of theory and data from semi-natural mesocosms shows that sex has a two-fold cost relative to asexual reproduction in the freshwater snail *Potamopyrgus antipodarum*. Yet asexual and sexual females coexist in nature. A strong selective force must therefore counterbalance the cost of sex. Previous work had shown that a sterilizing trematode, *Microphallus* sp., commonly infects *P. antipodarum* in natural populations. In Chapter 2, field surveys and experimental inoculations reveal substantial variation in both infection prevalence and in host susceptibility to local *Microphallus* around a single small lake. Variation in prevalence arises from variation in environmental factors and variation in susceptibility, a proxy for coevolutionary selection. Chapter 3 shows that the frequency of sexual females also varies substantially around this lake and is tightly positively correlated with susceptibility. Susceptibility can explain the majority of geographic variation in sex, far more than can infection prevalence. This result specifically points to the significance of

coevolutionary selection in the maintenance of sex. Chapter 4 provides support for the Red Queen that is taxonomically and conceptually unique. Applying phylogenetic comparative methods to the nematode phylum reveals that parasitic nematode taxa are more likely to be obligately outcrossing than their free-living relatives.

Variation in parasite virulence is another curious anomaly in evolutionary biology. Chapter 5 presents the results of experimental selection on reduced antagonism between a nematode host and its virulent bacterial parasite. Reduced antagonism evolved only when host and parasite were able to coevolve. This result argues that coevolution contributes to the evolution of virulence. Ultimately, this dissertation shows that coevolution lies at the root of some of evolution's most puzzling phenomena and can explain the maintenance of genetic and phenotypic diversity in natural populations.

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Introduction

“Now, here you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!”

~ Lewis Carroll, *Through the Looking Glass* (1897), page 50

The idea of coevolution coalesced in the 1960's (e.g. Mode 1958; Pimentel 1961; Ehrlich and Raven 1964; Janzen 1966) (reviewed in Thompson 1982). The term "coevolution" originally lacked a strong definition (Janzen 1980). Coevolution as a concept was thus loosely applied and indirectly tested (Thompson 1999). The study of coevolution, however, is emerging from these growing pains. We now have strong definitions. I rely upon John Thompson's: he defines coevolution as "reciprocal evolutionary change between interacting species driven by natural selection" (Thompson 2005). We also have specific, testable hypotheses. These include components of the geographic mosaic theory of coevolution (Thompson 1999) and contrasting modes of reciprocal selection (arms race vs. fluctuating selection dynamics). Lastly, we have experimental tools that enable us to observe and manipulate coevolution in the lab (Brockhurst and Koskella 2013). Thanks to the resulting theory and data, the significance of coevolution is undeniable. Throughout the natural world, we see that coevolution can impose strong reciprocal selection resulting in rapid evolutionary change.

My dissertation applies coevolutionary principles to two of evolutionary biology's most important problems: the evolutionary maintenance of sexual reproduction and the evolution of parasite virulence. In the sections below, I introduce both problems and summarize the contribution made by my dissertation chapters. Chapters 1 through 4 address the evolutionary maintenance of sex. In Chapter 1, I pair theory with experimental data to establish the problem: sex is costly. In Chapters 2 and 3, I use field observations and experimental manipulations to show that coevolutionary selection can explain the geographic distribution of sex in a natural population of freshwater snails. In Chapter 4, I apply comparative methods to show that coevolutionary selection can

explain the phylogenetic distribution of sexual outcrossing in nematodes. Chapter 5 addresses the evolution of parasite virulence. I use experimental coevolution of a nematode host and bacterial parasite to demonstrate that evolutionary transitions in antagonism require coevolution. As a whole, my dissertation attests to the unique power of coevolution.

Coevolution and the maintenance of sexual reproduction

Consider a sexual population in which a single sexual female produces a daughter with an unusual mutation. The mutation renders the daughter asexual. Instead of mating with a male to produce sons and daughters, she clones herself, making more asexual females. The daughter is equal to her mother in every other way: she occupies the same ecological niche, she can produce the same number of offspring, and her offspring have the same probability of surviving to reproduce. In this scenario, the mutation to asexuality will rapidly spread to fixation. The ancestral sexual lineage will disappear in tens of generations. Why? The answer is simple: males. John Maynard Smith first explained that investing in sons reduces the per-capita birth rate of sexual females. If 50% of a sexual female's offspring are sons, she has spent ~50% of her resources on offspring that are unable to bear children. Hence, by shunting all of those resources into making reproductive daughters, a rare asexual mutant can double in frequency each generation. This model is known as the two-fold cost of males (Maynard Smith 1971b, 1978).

Can such a simple model apply to natural populations? In particular, is the simplifying assumption, that asexual and sexual females are otherwise equal, realistic?

Chapter 1 answers these questions. Sexual and asexual morphs of the freshwater snail *Potamopyrgus antipodarum* coexist in lakes and streams throughout New Zealand. Prior studies of this snail showed that sexual and asexual females occupy the same ecological niche and produce equal numbers of eggs (Jokela et al. 1997a; Jokela et al. 1997b; Paczesniak et al. 2014). What we did not know for *P. antipodarum*, or for any system in fact, is if sexual and asexual females produce an equal number of surviving offspring (Meirmans et al. 2012). This assumption is the crux of the two-fold cost of males. In Chapter 1, we build upon Maynard Smith's original model to make testable predictions. Specifically, our expanded theory enabled us to predict how frequent asexual offspring should be given 1) how frequent their parents were in the prior generation and 2) how costly sex is. To experimentally test this prediction, we reared field-collected populations of *P. antipodarum* in semi-natural mesocosms. From these populations, we obtained the frequency of asexual parents and offspring. The cost of sex was then easily estimated. We found that our experimental data were consistent with a two-fold, or slightly greater, cost of sex. Assuming a sex ratio of 50% males in *P. antipodarum*, this result requires that asexual females make as many, or slightly more, surviving offspring than do sexual females. This work presents the first direct estimate of the cost of sex. Clearly, Maynard Smith's simple model has natural relevance.

A two-fold fitness cost is a rather high price to pay for any trait. Nor is the cost of males the only proposed cost of sex. Other potential costs of sex include the cost of meiosis (Williams 1971; Williams 1975), the cost of recombination (Charlesworth 1975), and the specific risks and energetic costs incurred through mating. One could then reasonably predict that a survey of the natural world would reveal sex to be an unpopular

strategy, with the vast majority of organisms reproducing asexually. The natural world, however, tells us precisely the opposite. The vast majority of eukaryotes reproduce sexually (Bell 1982; Suomalainen et al. 1987; Dacks and Roger 1999; Billiard et al. 2012). Obligate asexuality is exceedingly rare (fewer than 0.1% of animal species), and few of those lineages (if any) have persisted through evolutionary time (reviewed in Vrijenhoek 1998). In fact, asexual reproduction is so unusual that biologists in the 1800's were scandalized by the discovery that the unfertilized eggs of several arthropod taxa could develop. Rudolf Wagner, a prominent physiologist, described the phenomenon as "most distasteful" (as quoted by Churchill 1979).

Evolutionary theory thus failed to predict natural patterns. This mismatch led Graham Bell (1982) to name sex "the queen of problems," "the largest and least ignorable and most obdurate" of unknowns in evolutionary biology. Bell's statement still rings true today. Our relationship to the problem has changed though. In 1982, it was clear that strong selection must act to counterbalance the cost of sex. In his book *The Masterpiece of Nature* (1982), Bell outlined a profusion of largely untested hypotheses for what forces might apply that necessary selection. Since that time, many evolutionary biologists, Bell included, have whittled down the list with theory and data. Roughly two hypotheses have withstood their efforts. For proponents of one or the other hypothesis, a key point of contention is the significance of coevolutionary selection. In the following sections, I will review the hypotheses and discuss the contribution that Chapters 2 through 4 make to the field's ongoing debate. Ultimately, my work argues that the problem of sex requires careful study of the distribution of coevolutionary selection in natural populations.

Candidate 1, the Red Queen Hypothesis

Populations experience variation in their environment. This fact is perhaps the most salient feature of the natural world. Not surprisingly, environmental variation features prominently in reflections on the advantages of sex. What is surprising is that the maintenance of sex seems to require the environment to vary in a curious way. Temporal variation in and of itself does not maintain sex (G.C. Williams's lottery model 1973; 1975). Sex is more common in stable environments (e.g. the tropics) than in unpredictable environments (e.g. temperate zones; disturbed areas) (Levin 1975; Maynard Smith 1978; Bell 1982). Nor can spatial variation explain the observed distribution of sex (Bell's Tangled Bank 1982) (Burt and Bell 1987; Lively 1987). Rather, the models of Maynard Smith and others showed that the environment must be “bloody-minded” (Maynard Smith 1985). Imagine that environmental conditions strongly favor a single phenotype. The associated genotype increases in frequency. Only a few generations later though, conditions change. The very same phenotype is now specifically disfavored. Such fickle, fluctuating selection can counterbalance the costs of sex, because sexual lineages retain the genetic variation needed to continually adapt (Maynard Smith 1971b, 1978).

From where might such “bloody-minded” selection arise? Certainly no abiotic force commonly shifts in the rapid, specific way required by the models. In time, researchers began to consider the biotic world (Hamilton 1975; Levin 1975; Jaenike 1978; Hamilton 1980). Selection by coevolving antagonists meets our criteria. Let's assume that natural enemies (e.g. parasites) specifically attack one, or a subset, of the host genotypes in a population. Host genotypes that have a fitness advantage will increase

in frequency, becoming common. The most successful enemies are then those that specifically attack these common host genotypes (i.e. the most abundant resource). The biotic environment now specifically selects against these formerly fit genotypes (Jaenike 1978; Hamilton 1980; Hamilton et al. 1990). We know this model as the Red Queen hypothesis (Bell 1982).

The Red Queen hypothesis makes clear predictions that can be tested through experiments and field observations. A key prediction is that sex should be maintained in the presence of coevolutionary selection. A controlled laboratory experiment verified that coevolving parasites are indeed capable of maintaining sexual outcrossing in the nematode host *Caenorhabditis elegans* (Morran et al. 2011). Coevolution is critical: selfing rapidly invaded when parasites were present but unable to coevolve (Morran et al. 2011; Slowinski et al. 2016).

So coevolution can maintain sex in the lab – but does it work in nature? It is difficult to measure coevolutionary selection in natural populations, much less to isolate it from the many other selective forces that may be operating. The Red Queen has nonetheless garnered a remarkable amount of support from natural populations. In multiple systems, we see that common host clones become disproportionately infected by local parasites (Lively et al. 1990; Chaboudez and Burdon 1995). They are subsequently driven down in frequency (Dybdahl and Lively 1998; Lively and Dybdahl 2000; Jokela et al. 2009; Koskella and Lively 2009; Wolinska and Spaak 2009). Antonovics and Ellstrand (1984) and Ellstrand and Antonovics (1985) found evidence of rare advantage in sweet vernal grass, possible mediated by parasites (Schmitt and Antonovics 1986; Kelley 1993; Kelley and Shykoff 1994). Accordingly, selfing and asexual reproduction

are most common in areas where the prevalence of parasitism is low (Levin 1975; Glesener and Tilman 1978; Bell 1982; Schrag et al. 1994; Kumpulainen et al. 2004; Verhoeven and Biere 2013). We can infer that sexual reproduction is maintained where coevolutionary selection is strong.

Is coevolution a general explanation for the maintenance of sex? The Red Queen makes several key assumptions, including: 1) high virulence (May and Anderson 1983; Howard and Lively 1994; Otto and Nuismer 2004) and 2) a specific form of infection genetics (Jaenike 1978; Clay and Kover 1996; Blanford et al. 2003; Otto and Nuismer 2004). These assumptions are considered too restrictive to apply generally in nature (though see Agrawal and Lively 2002, 2003; Salathé et al. 2008; Engelstadter and Bonhoeffer 2009; Lively 2009). However, we have only studied coevolution, virulence, and infection genetics in a handful of natural systems. It is said that “diseases are like the stars. The longer you look the more you see” (Anonymous, from Antonovics et al. 2011). The same may well be true for virulent, coevolving parasites.

Candidate 2, Hill-Robertson interference

Without recombination, mutations at different loci become stuck together. Good mutations may be stuck with bad mutations, or bad with bad. Such negative genetic associations (“Hill-Robertson effects”) arise most frequently in small populations (Hill and Robertson 1966), by chance or by selection. They limit genetic variation for fitness, impeding the response to directional selection (Charlesworth et al. 2009). This argument is the conceptual backbone of the earliest hypotheses for the maintenance of sex.

Weismann (Weismann, 1904) first proposed that sexual recombination serves to increase variation (from Burt 2000). Thus sexual lineages persist over longer time scales than asexual lineages. Fisher (1930) and Muller (1932) formalized his argument. Muller's Ratchet later proposed that, in finite populations, asexual lineages go extinct as they accumulate deleterious mutations over time (Muller 1964). All of these models were rejected as stand-alone explanations for sex. Asexual lineages have such an advantage that they should fix in populations before the proposed advantages of sex are realized (Williams 1975; Maynard Smith 1978).

“Hill-Robertson interference” is the modern incarnation of these early hypotheses (Felsenstein 1974). Much theory shows that increased recombination is favored because it disrupts the negative linkage disequilibrium that arises from hitchhiking, background selection, or simply chance (e.g. Barton 1995b; Barton and Otto 2005; Keightley and Otto 2006; Roze and Barton 2006; Hartfield et al. 2010). These studies feature directional selection on recurrent deleterious and/or beneficial mutations. Initial work suggested that this process would only favor recombination in small populations, where the effect of drift is strongest (Barton and Otto 2005; Roze and Barton 2006). If selection acts at many linked loci, however, recombination may be favored in larger populations (Otto and Barton 2001; Iles et al. 2003; Keightley and Otto 2006) (reviewed in Hartfield and Keightley 2012).

The Red Queen hypothesis and Hill-Robertson interference differ on many points. First, Hill-Robertson theory primarily address selection for recombination (Hartfield et al. 2012), while Red Queen studies often address selection for sex (Peters and Lively 1999; Otto and Nuismer 2004). Though the two are certainly linked, it is not clear that

recombination has the same costs and benefits as sex (Bell 1982; Peters and Lively 2007). Second, Hill-Robertson interference invokes directional selection (Hill and Robertson 1966; Felsenstein 1974), while frequency-dependent selection and fluctuating epistasis underpin the Red Queen (Barton 1995a; Peters and Lively 1999). Third, the two hypotheses differ in the degree to which they emphasize the origin of that selection. The Red Queen is an ecological hypothesis. Ecological hypotheses are intimately concerned with relating sex to the selective forces that might act upon it. For the Red Queen, the selective force of interest is natural enemies. Hence, empirical tests can specifically test the link between an ecological force (e.g. coevolving parasites) and sex. In contrast, selection derives from an abstract ecological force in Hill-Robertson models (Otto 2009). The implication is that Hill-Robertson interference is more general than the Red Queen.

There is much potential for common ground. Selection by coevolving parasites may periodically shrink the effective population size of clonal lineages. The effect of genetic drift could then be strong enough to drive clonal lineages extinct (Howard and Lively 1994; Howard and Lively 2002; Galvani et al. 2003). Negative genetic associations may arise from interference between different types of selection. For example, frequency-dependent selection on infection loci could conflict with directional selection on linked loci unrelated to infection (Hodgson and Otto 2012). Both hypotheses together may explain the ubiquity of sex (West et al. 1999).

At this point, support for Hill-Robertson interference is largely theoretical, but several lab studies do support its general thrust. Populations with recombination adapt to novel selection pressure better and faster than populations without recombination (e.g. McPhee and Robertson 1970; Malmberg 1977; Rice and Chippindale 2001; Morran et al.

2009). McDonald et al. (2016) provided direct evidence that rare recombination events accelerate adaptation by alleviating genetic interference in the yeast *Saccharomyces cerevisiae*. Increased recombination also evolves in response to long-term directional selection (Flexon and Rodell 1982; Burt and Bell 1987; Korol and Iliadi 1994). Poon and Chao (2004) linked recombination to genetic drift: recombining bacteriophage lines outperformed non-recombining lines under directional selection at low, but not high, effective population sizes. To my knowledge, there is a single study that directly applies Hill-Robertson predictions to natural populations. Ross et al. (2013) found that asexuality in scale insects was positively correlated with host range. The authors used host range as a proxy for effective population size. Clearly, the field must better evaluate the relevance of Hill-Robertson interference to the maintenance of sex in natural populations. The first step is to generate falsifiable predictions.

Chapters 2 and 3: the biogeography of sex and coevolution

The Red Queen hypothesis and Hill-Robertson interference differ in the significance that they place upon coevolutionary selection. Hence, we must evaluate the degree to which coevolutionary selection can explain the geographic distribution of sex. This is the goal of Chapters 2 and 3.

The freshwater snail *P. antipodarum* is commonly infected by the sterilizing trematode parasite *Microphallus*. We can measure susceptibility to *Microphallus* in the lab. We first expose a group of hosts to a fixed dose of *Microphallus* and then measure the proportion of infected hosts. We know that susceptibility is rooted in the interaction

of coevolving host and parasite genotypes (Dybdahl and Krist 2004; Krist et al. 2004; Dybdahl et al. 2008; Koskella et al. 2011). For example, susceptibility is highest when hosts are exposed to local, coevolving parasites (Lively 1989; Lively and Dybdahl 2000; Lively et al. 2004). Susceptibility can thus serve as a measure of coevolutionary selection.

In Chapter 2, we show that the prevalence of *Microphallus* infection varies dramatically around the shoreline of a small lake. One-third of this variation in prevalence arises from variation in the susceptibility of hosts to the lake's local parasites. The remaining two-thirds of the variation is likely due to variation in exposure. This study is a rare demonstration of the significance of genetic variation and coevolution to variation in infection prevalence (Gibson et al. 2016b).

Chapter 2 also suggests that infection prevalence may be an inaccurate measure of coevolutionary selection. Prevalence is only partly rooted in coevolutionary genetics (susceptibility). Therefore, infection prevalence should be an inaccurate proxy for testing the relationship between coevolutionary selection and sex. This problem is general – infection prevalence typically has a major environmental component that may obscure any relationship between prevalence and coevolution (Grosholz 1993; Duffy et al. 2012; Altman and Byers 2014; Jousimo et al. 2014). Yet prevalence is the only proxy used in tests of the Red Queen (e.g. Johnson 1994; Ben-Ami and Heller 2005; Meirmans et al. 2006b; Verhoeven and Biere 2013). In Chapter 3, we target variation in coevolutionary selection by instead using susceptibility as a proxy. We find that susceptibility is tightly positively correlated with the frequency of sexual females around our small study lake. Susceptibility can in fact explain the vast majority of variation in sex. Consistent with our

a priori prediction, the distribution of sex is more closely linked to susceptibility than to prevalence (Gibson et al. 2016c).

Chapters 2 and 3 strongly support a specific role for coevolution in the maintenance of sex in *P. antipodarum*. There is limited support for other hypotheses in this system. No other ecological variable is clearly associated with sex (Lively 1987; Lively 1992; Jokela et al. 1997a; Jokela et al. 2003). In Chapter 3, sex was more tied to the spatial distribution of susceptibility than to that of other environmental covariates. There is no evidence that genetic drift is a significant force in the lake-wide population, or even at individual sites. The effective population size of *P. antipodarum* is too large for genetic drift to be a factor (Paczynski et al. 2014). Mutation accumulation in asexual lineages could contribute to the maintenance of sex, if parasites drive clonal lineages through periodic bottlenecks (Howard and Lively 1994). Field surveys and experiments suggest that formerly common clones can fall to low frequencies following over-infection by coevolving parasites (Jokela et al. 2009). Clonal lineages also have elevated mutation rates relative to sexual lineages (Neiman et al. 2010).

In Chapter 3, we make a case for careful consideration of coevolution. Agents of the Red Queen require genetic specificity and severe fitness consequences. Host-parasite interactions are thus uniquely qualified. We should not, however, lose sight of the fact that, traditionally, the Red Queen is about antagonistic coevolution, not parasites. Not all host-parasite interactions are coevolving. Given the current state of theory, tests of the Red Queen hypothesis cannot be interpreted without a coevolutionary framework. This is problematic in the field: evaluating coevolution can be challenging. It is also problematic

in the lab: the Red Queen hypothesis should be tested using host-parasite interactions with a coevolutionary history.

Chapter 4: the parasite's perspective

Parasites are often overlooked in favor of their charismatic hosts. Yet parasites in particular should experience strong coevolutionary selection. Indeed, selection is expected to be stronger on obligate parasites: failure to infect means death (Howard and Lively 2002; Galvani et al. 2003; Salathé et al. 2008; King et al. 2011b). Hence the reproductive strategies of parasites are an interesting, though largely unexplored, realm for the Red Queen.

Bell (1982) first formulated the Red Queen hypothesis's prediction for parasite reproduction: "in comparable taxa, the Red Queen predicts that thelytoky should be more common in free-living than in parasitic forms" (pg. 381). We test this prediction in Chapter 4. Nematodes show unusual variation in ecology and reproductive mode. Parasitism has arisen multiple times on free-living lineages (Blaxter et al. 1998; Dorris et al. 1999; De Ley 2006; van Megen et al. 2009). Selfing and asexual reproduction (thelytoky) have arisen multiple times on outcrossing lineages (Kiontke et al. 2004; Kiontke and Fitch 2005; Cutter et al. 2008; Denver et al. 2011; Kiontke et al. 2011a). We use phylogenetic comparative methods to test for correlations between these evolutionary transitions. As predicted, outcrossing is significantly correlated with parasitism of animal hosts. We find that selfing and asexuality evolve far more readily on free-living than on animal parasitic lineages. Interestingly, outcrossing is not correlated with parasitism of

plant hosts. Our results argue that the Red Queen is an explanation, at least in part, for the macroevolutionary distribution of outcrossing in nematodes (Gibson and Fuentes 2015).

This study cannot differentiate the Red Queen hypothesis from Hill-Robertson interference. Nematodes may experience population bottlenecks upon infecting animal hosts. We would then predict a smaller effective population size for animal parasites vs. free-living nematodes. The limited theory on parasite reproductive modes has in fact argued that outcrossing is maintained by the joint effect of coevolution and processes linked to drift (Howard and Lively 2002; Galvani et al. 2003). We could weigh the relative contribution of coevolution with follow-up research on plant parasitic nematodes. These taxa may vary in effective population size: some colonize their host externally, and so perhaps have larger effective population sizes than do internal parasites. Plant parasites may also vary in the strength of coevolutionary selection: some species may only coevolve weakly with their hosts due to extremely diffuse host ranges and/or predominantly agricultural associations. These nematodes thus show great promise for more direct assessments of the significance of coevolutionary selection.

Chapter 4 makes clear the far-reaching implications of the Red Queen hypothesis. It suggests that coevolution maintains genetic variation in parasitic lineages. In turn, genetic variation in parasitic lineages can increase the potential for disease spread. We must then consider incorporating coevolutionary genetics into epidemiological models, as in Lively (2010a; 2016) and King and Lively (2012). Genetic variation in parasitic lineages can also increase the response to selection on virulence or drug resistance. In the following section, I discuss my work linking coevolution to virulence.

Coevolution and the evolution of virulence

Why would a parasite harm its host? Degrading one's shelter and food supply cannot possibly be favored in the long-term. Virulence is thus maladaptive, arising only in the earliest unions of a host and parasite. Or so the "conventional wisdom" goes (Alexander 1981; Palmieri 1982).

Let's test out this logic. Imagine a host infected by two different parasites. Parasite A multiplies relatively slowly. Thus it has little negative affect on the fitness of its host. Parasite B multiplies rapidly, colonizing and degrading much of the host. It severely reduces host fitness. Which is the better strategy? The conventional wisdom would argue that parasite A's prudent strategy is adaptive. Yet parasite A has produced few transmissible stages by the time that the host dies due to parasite B's rapacious growth. Parasite B has produced plenty of transmission stages. Assuming susceptible hosts are readily available, parasite B will spread rapidly in the host population. Prudent parasite A will go extinct. Just as we see for the problem of sex, the strategy that is best for the group is not always the victor in a battle between individuals. Sexual reproduction and low virulence may have long-term benefits, but they can be invaded by rapidly growing asexual or virulent lineages. Thus the conventional wisdom was overthrown, for both sex and virulence.

The argument above is based upon the trade-off hypothesis of virulence evolution (Anderson and May 1982; Ewald 1983). This hypothesis models virulence as a trade-off between transmission and infection longevity. Virulence is positively correlated with the production of offspring, or transmission stages. Virulence is also positively correlated

with host mortality, which curtails the period of parasite reproduction. The trade-off hypothesis thus predicts that intermediate virulence maximizes parasite virulence (May and Anderson 1983). With the formulation of this hypothesis, the field of virulence evolution flourished. Today, we have many more testable hypotheses and much experimental data. In Chapter 5, we propose and test a new hypothesis: coevolution is critical to the evolution of parasite virulence.

The trade-off hypothesis links virulence to transmission mode. A horizontally transmitted parasite jumps between hosts. As long as more susceptible hosts are nearby, a horizontally transmitted parasite can afford to harm or kill its host. Covariance of host and parasite fitness can be negative. A vertically transmitted parasite moves from parent to offspring, so transmission requires host reproduction. The covariance of host and parasite fitness is positive. The direction of selection on virulence should therefore vary with transmission mode (Anderson and May 1982; Ewald 1987; Bull 1994; Frank 1996; Lipsitch et al. 1996; Wade 2007). In many experimental tests, vertical transmission indeed selects for reduced parasite virulence relative to horizontal transmission (Bull et al. 1991; Herre 1993; Clayton and Tompkins 1994; Turner et al. 1998; Messenger et al. 1999; Stewart et al. 2005; Sachs and Wilcox 2006).

In Chapter 5, we argue that these experimental tests confound transmission mode with coevolution. Under horizontal transmission, parasites continually shift between host lineages. There is little potential for coevolution. Under vertical transmission, a parasite and host lineage are paired for multiple generations. Tight coevolution is likely. Thus the potential for coevolution also varies with transmission mode. Does coevolution contribute to the evolution of reduced virulence?

We tested this hypothesis with experimental coevolution of the nematode *Caenorhabditis elegans* and its lethal bacterial parasite *Serratia marcescens*. Evolutionary transitions in virulence are relevant to this association. *S. marcescens* strains vary substantially in virulence to *C. elegans*. *Serratia* species are also cuticular or gut mutualists to rhabditid nematodes, including caenorhabditids (Petersen and Tisa 2013). We manipulated the potential for coevolution while selecting for reduced antagonism between *C. elegans* and *S. marcescens*. We use the term “antagonism” here to emphasize the joint nature of the phenotypes of interest. Though virulence is not exclusively a parasite trait, it is commonly assumed to be. Our results demonstrate that coevolution contributes to the evolution of reduced antagonism (i.e. reduced virulence). After 20 generations of selection, reduced antagonism evolved only when coevolution was possible. Independent selection on either partner failed to produce evolutionary change. Potentially coevolving lines were strongly locally adapted for reduced antagonism, further underlining the significance of coevolution. Our study is the first to directly test the relationship between coevolution and virulence (Gibson et al. 2015).

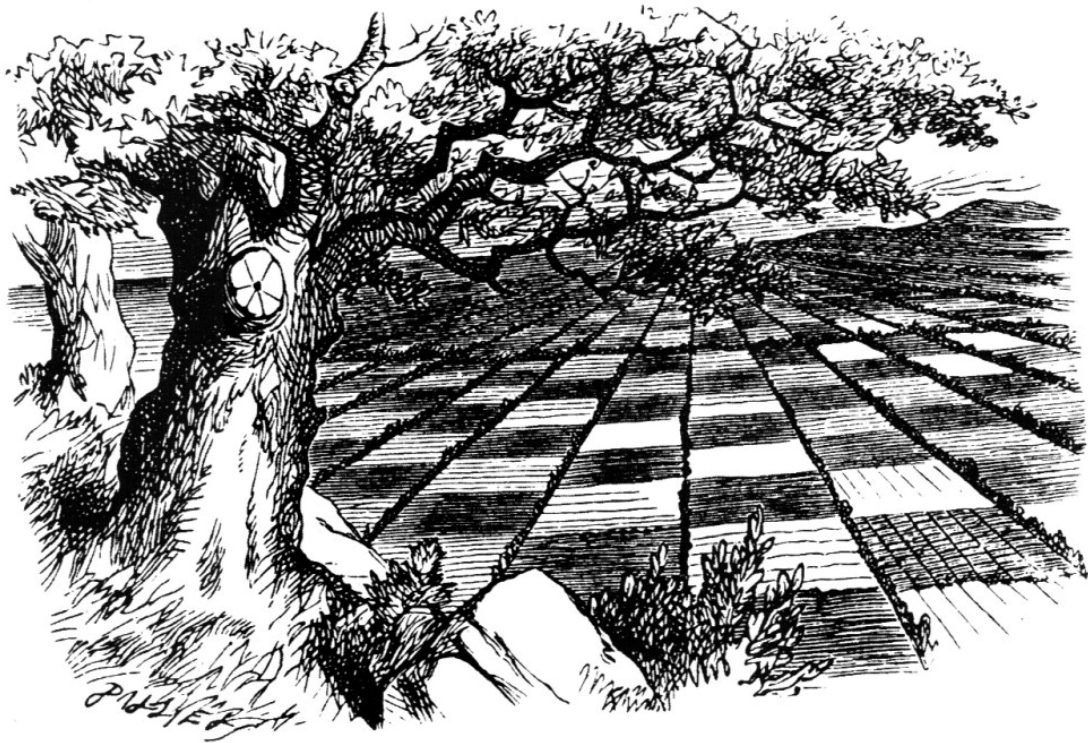
We can see hints of such a link in earlier literature. Several authors make note of the contextual nature of virulence (Traub 1939; Bull et al. 1991; Bull and Molineux 1992). In experimental pairings, a parasite genotype’s virulence depends upon its host genotype. Coevolution can also promote virulence. Strains of *S. marcescens* were selected for increased virulence, with and without coevolution. Those selected under coevolution killed ancestral hosts much faster than other evolved strains (Morran et al. 2011). There is clearly progress to be made by incorporating coevolution into models of virulence evolution. The rise of experimental coevolution also makes coevolution an

experimentally tractable process to study in multiple systems. This avenue may be of particular interest given that many of the modern world's most concerning infectious diseases are ones for which the host and parasite do not share a coevolutionary history.

Coevolution: oh, the places you'll go

So, is coevolution going anywhere? The results of my dissertation research offer an emphatic yes. My work, and that of many others, shows that the coevolutionary process lies at the root of the most puzzling phenomena in evolutionary biology. A rigorous theoretical framework now allows us to pose many new questions. A rich array of experimental techniques means we may even be able to answer them. Much current research focuses on simple coevolutionary interactions in controlled laboratory settings. To what extent can we extrapolate these lab-based findings to natural populations? We do not currently know how important coevolution is relative to other evolutionary forces (e.g. evolution of sex – see discussion above). Moreover, the complexity of interactions in natural communities may fundamentally alter the coevolutionary process. These problems can only be addressed through the study of natural populations. In addition, evolutionary ecology is now obsessed with host-associated microbial communities. Productive growth of this field requires careful application of coevolutionary principles and experimental techniques (as in Koskella 2013). In turn, the diversity and complexity of interactions in these communities calls for an expansion of current coevolutionary theory. Finally, applied problems may find creative solutions in coevolutionary principles. Clear connections exist to control of agricultural parasites (Thompson 2005;

Brown and Tellier 2011) and to management of drug resistance (e.g. Pal et al. 2007). Though a citizen of the Red Queen's curious country, perhaps coevolution is indeed getting somewhere.



The Red Queen's curious country. Illustration by John Tenniel for Lewis Carroll's 1871 *Through the Looking Glass, and What Alice Found There*, Chapter 2. In the public domain (PD-1923). Image obtained from Wikimedia Commons.

Chapter 1

The two-fold cost of sex: experimental evidence from a natural
system

Gibson, Delph, & Lively

*“Consider first the twofold disadvantage of producing males. Although
absurdly simple, the point is so fundamental and so often
misunderstood...”*

~ John Maynard Smith, *The Evolution of Sex* (1978), page 2

Introduction

Over four decades ago, Maynard Smith showed that a mutation to asexual reproduction would rapidly spread to fixation in an otherwise sexual population (Maynard Smith 1971a; Maynard Smith 1978). His reasoning was that the production of sons by sexual females reduces the per-capita birth rate of the sexual population. Hence, there is a cost of sexual reproduction that Maynard Smith called the “cost of males.” Assuming all else is equal, the cost of sex can be two-fold in outcrossing populations with separate sexes and equal sex ratios. This simple model led to one of the most interesting questions in evolutionary biology: why is there sex? There are, however, no direct estimates of the proposed cost of sex. Here we generalized the model in order to predict the change in frequency of asexuals for any cost of sex and for any starting frequency of asexuals. We then quantified the cost of sex in a natural, mixed population of sexual and asexual snails using experimental evolution in large, replicated outdoor mesocosms. Consistent with the “all-else equal” assumption, we found that the increase in the frequency of asexual snails closely matched that predicted under a two-fold cost. Our direct estimate of the cost of sex thus supports Maynard Smith’s original model. Hence, for sex to be maintained in natural populations, there must be strong selection favoring sexual over asexual reproduction.

The cost of males (along with Williams’ “cost of meiosis” (1971; 1975)) sparked an enduring paradox in evolutionary biology: if sexual reproduction is so much more costly than cloning, why is it so common? According to the two-fold cost of sex, asexual mutants should fix rapidly in a population, well before the long-term disadvantages of clonal reproduction can surface. Sex, however, abounds. This inconsistency between

theory and nature instigated the ongoing hunt for forces that can counterbalance the short-term costs of sex.

Although the cost of sex is the foundation of the paradox of sex, there are no direct experimental measures of the cost. Several studies have addressed the critical assumption of Maynard Smith’s model, known as the “all-else-equal” assumption. This assumption is simply that sexual and asexual females are equally fecund and that the survivorship of their offspring is equal (Fig. 1a). There is mixed support for this assumption (Meirmans et al. 2012): asexual and sexual individuals have similar fecundity and/or offspring survival in five of ten cases (e.g. snails (Jokela et al. 1997b; Crummett and Wayne 2009); rotifers (Stelzer 2011); see also (Meirmans et al. 2006a) on dandelions). A few studies have taken an additional step by showing that the frequency of asexual individuals increases over time in mixed populations, arguing that sex is indeed costly in some systems (Browne and Halanych 1989; Jokela et al. 1997b; Stelzer 2011). A crucial question remains, however: exactly how costly is sex?

Results and Discussion

To address this question, we first constructed a general model that predicts the frequency of asexual individuals in the next generation, for any cost of sex and any initial frequency of asexuals. From basic population genetic theory (Gillespie 1998), we can write the frequency of asexuals in the next generation (q_{t+1}) as:

$$q_{t+1} = q_t \frac{W_{asex}}{\bar{W}} \quad (1)$$

where q_t is the starting frequency, W_{asex} is the per-capita birth rate for asexual females, and \bar{W} is the mean per-capita birth rate for the mixed population of sexual and asexual individuals:

$$\bar{W} = q_t W_{asex} + (1 - q_t) W_{sex} \quad (2)$$

Here W_{sex} is the per-capita birth rate for the sexual population. Let the per-capita birth rate of the sexual population be a fraction ($1/c$) of the asexual birth rate, where c represents the cost of sex. Therefore,

$$W_{sex} = \frac{W_{asex}}{c} \quad (3)$$

An estimated value of two for c would be consistent with a two-fold cost of sex, while c equal to one would mean that sexual females incur no cost. By substituting, equation (1) becomes:

$$q_{t+1} = \frac{c q_t}{1 + q_t(c - 1)} \quad (4)$$

Dividing both sides by q_t , we can calculate the fold-increase in the frequency of asexuals as:

$$\frac{q_{t+1}}{q_t} = \frac{c}{1 + q_t(c - 1)} \quad (5)$$

Given a two-fold cost, equation (5) illustrates that the proportional increase in asexual frequency declines from two to one as the frequency of asexuals (q_t) moves from rarity to fixation (Fig. 1b). Finally, equation (4) can be rearranged to directly estimate the cost of sex from any starting frequency of asexuals:

$$c = \frac{q_{t+1}(1 - q_t)}{q_t(1 - q_{t+1})} \quad (6)$$

In this model, the variable c represents the total cost of sex, which includes the cost of males weighted by any fecundity-survival asymmetries in sexual vs. asexual females. We represent these asymmetries using the variable r , which gives the ratio of the mean number of surviving offspring produced by asexual females divided by the mean number of surviving offspring produced by sexual females. A value of one for r would indicate that the all-else-equal assumption is met: sexual and asexual females produce the same number of surviving offspring. Let the variable s be the frequency of daughters produced by sexual females (i.e. the primary sex ratio). The cost of males is then $1/s$. The total cost of sex is simply the product of the cost of males and the female fecundity-survival ratio (Fig. 1a):

$$c = \frac{1}{s}r \quad (7)$$

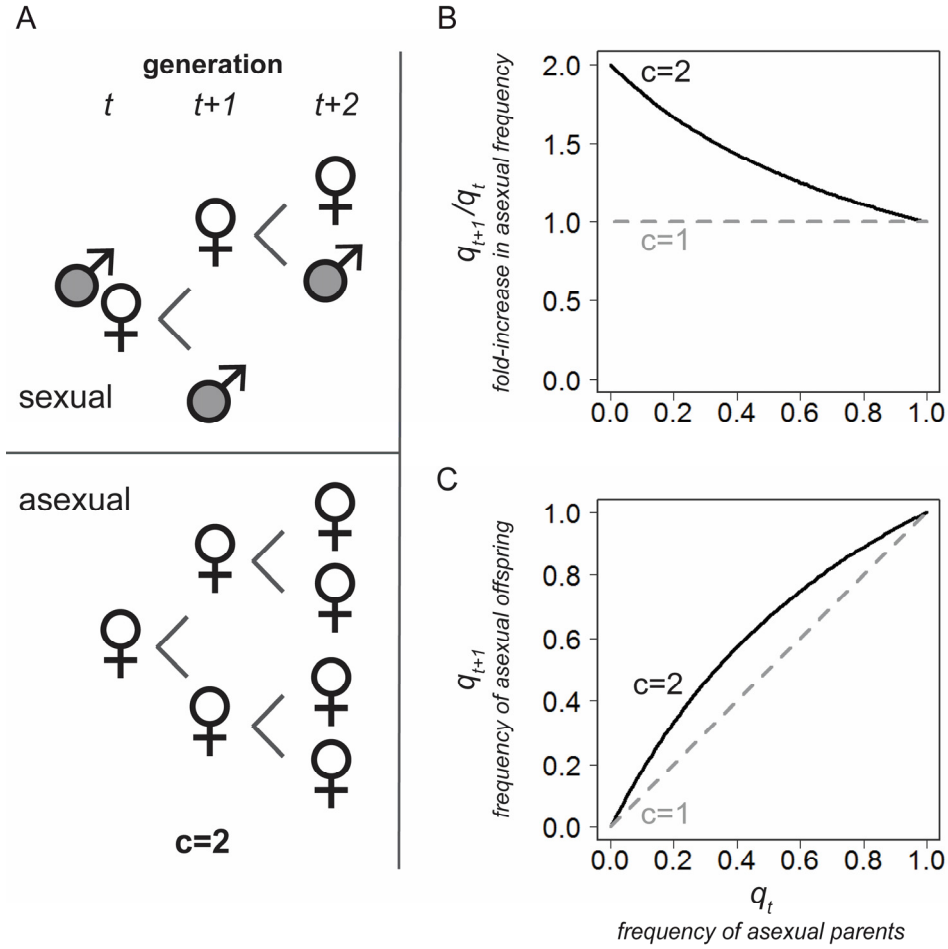
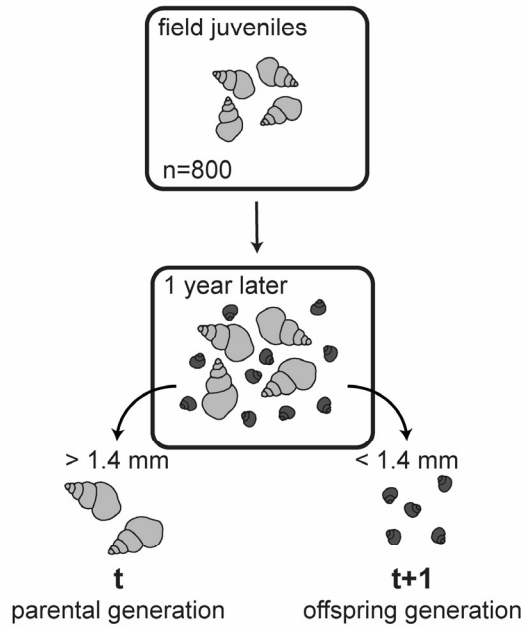


Figure 1: Theoretical predictions for the cost of sex. (A) Under a two-fold cost of sex ($c=2$), asexual females can produce twice as many childbearing offspring (females). The cost c is the product of the female fecundity-survival ratio r and the cost of males. Here, sexual and asexual females produce an equivalent number ($n=2$) of surviving offspring (fecundity-survival ratio, $r = 1$), consistent with the all-else-equal assumption. Sexual females make 50% daughters ($s = 0.5$), so the cost of males is two ($1/s = 2$) and the total cost of sex is two ($c = r * 1/s$). (B) Equation (5) shows that, under a two-fold cost ($c=2$, black solid line), doubling is observed only at low starting frequencies of asexual individuals. The proportional increase in asexual frequency declines from two to one as the frequency of asexuals in the parental generation (q_t) increases from rarity to fixation. This result is intuitive: the capacity to double becomes constrained as the starting frequency increases. Equation (4)'s corresponding prediction for the frequency of asexual individuals in the offspring generation (q_{t+1}) is shown in (C). We use equation (4) when fitting models to experimental data. When sexual reproduction is not costly ($c=1$, gray dashed line), asexuals have no intrinsic birth rate advantage and will not change in frequency from parental to offspring generations.

We used this extension of Maynard Smith's model to directly measure the cost of sex in the New Zealand freshwater snail *Potamopyrgus antipodarum*. Sexual lineages coexist with asexual lineages, which arise by mutation from local sexual genotypes (Neiman et al. 2005) and are primarily triploid females (sexuals are diploid) (Neiman et al. 2011). Offspring develop in their mother's brood pouch, facilitating life-history comparisons. The all-else-equal assumption is met for fecundity in *P. antipodarum*: sexual and asexual females are similar in size at reproductive maturity, brood at similar rates, and have an equal number of eggs per brood (Jokela et al. 1997a; Jokela et al. 1997b; Paczesniak 2012) (Supporting Information). We did not know whether sexual and asexual females are equally likely to survive to reproduction, or if they produce an equivalent number of viable offspring. We tested this assumption in the present study. There is direct evidence that sex can be costly in this species. Under competition with a sexual lineage in experimental populations, a single clone increased from a starting frequency of 35% to 62% in a few generations (Jokela et al. 1997b).

To directly quantify the total cost of sex, c , we added 800 juvenile snails sampled from four sites at Lake Alexandrina (South Island, New Zealand, Fig. S1a) to six 800-liter mesocosms (Fig. S1b). By using field collections, we maintained the relative frequencies and genetic diversity of clonal and sexual lineages present in the natural population. This is important, as the asexual population of snails is known to consist of many genetically distinct clones (Dybdahl and Lively 1995). The snails matured and reproduced over the course of one year. We then separated parents and offspring by size into discrete generations, and we estimated the frequency of asexuals in the parents (q_i)

A. Experimental mesocosms



B

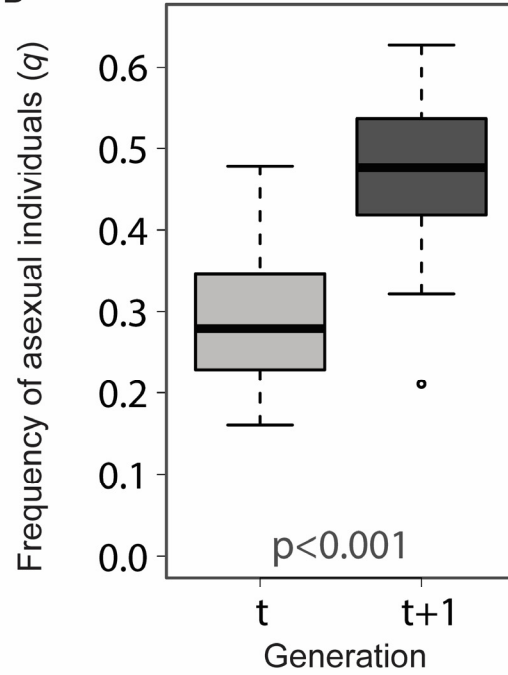


Figure 2: Increase in asexual frequency in experimental mesocosms. (A).

Mesocosms were initiated with 800 field-collected juveniles (grey), which matured to adulthood and produced offspring (black) over the course of one year. Parents (originally juveniles) and offspring were separated by size and split into discrete generations (t and $t+1$, respectively). We then estimated the frequency of asexual individuals in parent (q_t) and offspring (q_{t+1}) generations. (B) The frequency of asexuals increased from the parent (t) to offspring ($t+1$) generation. Box plot shows median (black bar), upper and lower quartiles (limits of box), minimum and maximum (whiskers, excluding outliers), and outliers (dots). Measure of significance is derived from the logistic model reported in the text. Each generation is represented by 18 mesocosms. The numbers of triploid females represented by each mesocosm are: 28.00 ± 1.61 SEM for parents and 23.67 ± 3.60 for offspring for the six mesocosms in 2012; 21.00 ± 1.97 for parents and 37.00 ± 3.29 for offspring in 2013 mesocosms; and 16.67 ± 2.75 for parents and 34.33 ± 2.03 for offspring in 2014 mesocosms.

and the offspring (q_{t+1}) (Fig. 2a). We conducted the experiment in three different years, for a total of 18 independent replicates.

Averaged over all 18 replicates, the frequency of asexuals increased 1.56-fold (95% CI [1.33, 1.79]) from parent to offspring generations (Fig. 2b; logistic model, generation: likelihood ratio $D = 109.7$, $df=1$ $p<0.001$). There was no variation in the direction of change between years (interaction: $D = 1.70$, $df=2$, $p=0.440$), but the overall frequency of asexuals was higher in the latter two years of the experiment (odds ratio vs. 2012: 2013= 1.73 [1.31, 2.31], 2014 = 1.61 [1.21, 2.15]; year: $D = 29.1$, $df=2$, $p<0.001$).

Using equation (4), we could then ask: is a two-fold cost the best approximation to our experimental data? We formulated four candidate models: (1) no cost of sex ($c=1$), (2) a two-fold cost ($c=2$), (3) the maximum likelihood estimate (MLE) of the cost, and (4) the MLE of costs that vary with year. We proposed model 4 because the composition of clones and/or the accuracy of the experiment may have differed among years. We used standard maximum likelihood procedures to estimate parameters and select the best model(s) for our experimental measures of q_t and q_{t+1} . Table 1 presents each model in the form of equation (4). We calculated AIC_c (Akaike information criterion for small sample sizes) and ranked models according to their ΔAIC_c values, which is the difference in the AIC_c values of the focal model and the best model (lowest AIC_c). The best model then has a ΔAIC_c value of zero, and values below two are consistent with substantial support. We calculated Akaike weights, w , to estimate the relative weight of evidence for each model (Burnham and Anderson 1998). Values of w near 0 indicate that a model is very unlikely to be the best model in the set of candidate models.

Table 1: Results of model inference and selection. We proposed four candidate models for our experimental data. These four models assume different values of the cost of sex: (1) no cost ($c=1$); (2) a two-fold cost ($c=2$); (3) the maximum likelihood estimate (MLE) of the cost; and (4) the MLE of costs that vary with year. Each model is represented in the form of equation (4). We ranked models according to ΔAIC_c and evaluated the weight of evidence for each model using w , the Akaike weight.

	Model ¹		logL	Parameters ²	AIC _c	ΔAIC_c ³	w ⁴
1	<i>No cost</i> $c = 1$	$q_{t+1} = q_t$	-76.61	1 (θ)	155.47	21.41	0.00
2	<i>2-fold</i> $c = 2$	$q_{t+1} = \frac{2q_t}{1 + q_t}$	-65.91	1 (θ)	134.06	0.00	0.71
3	<i>Estimate</i> $c = MLE$	$q_{t+1} = \frac{cq_t}{1 + q_t(c - 1)}$	-65.58	2 (θ, c)	135.97	1.90	0.27
4	<i>By year</i> $c =$ <i>MLE*year</i>	$q_{t+1} = \frac{cq_t}{1 + q_t(c - 1)}$ 2012: $c = c_0$ 2013: $c = c_0 + d_2$ 2014: $c = c_0 + d_3$	-64.93	4 (θ, c_0, d_2, d_3)	140.93	6.87	0.02

¹ “MLE” = maximum likelihood estimate. For model 4, the cost was indexed by experimental year. Maximum likelihood estimates of d_j that significantly deviate from 0 indicate that the cost of sex in experimental year j differed from that estimated in 2012 (Supporting Information).

² Total number of estimated parameters. To fit models to experimental data, we assumed a beta-binomial distribution for the likelihood functions and thus estimated an additional overdispersion parameter θ . The beta-binomial distribution is appropriate when data are overdispersed under the binomial distribution.

³ Used to rank models according to their level of support. $\Delta AIC_c = 0$ for the best model. Rule of thumb interpretation: $\Delta AIC_c < 2$ substantial support, $4 < \Delta AIC_c < 7$ considerably less support, and $\Delta AIC_c > 10$ no support.

⁴ The weight of evidence for a model, with a high of 1 and a low of 0.

From our candidate models, a two-fold cost of sex (model 2) and the maximum likelihood estimate of the cost (model 3) were the best approximations to our data (Table 1), having low ΔAIC_c and a high weight of evidence in their favor. The likelihood of model 3 was maximized at a cost of sex that slightly exceeds two ($c = 2.23$, 95% CI [1.82, 2.69]), but not significantly so (Fig. 3). We therefore concluded that the observed increase in the frequency of asexual *P. antipodarum* from generation t to $t+1$ in our experimental mesocosms was consistent with a two-fold cost of sex.

Based upon the magnitude of model 1's deviation from the best model and its low weight of evidence, the analysis firmly rejected model 1's assumption of equivalent per capita birth rates of sexual and asexual populations (i.e., no cost of sex). There was also little support for temporal variation in the cost of sex: the ΔAIC_c for model 4 is relatively large, exceeding the upper limit for our 95% confidence set of models ($\Delta\text{AIC}_c = 3.73$), and the weight of evidence is close to 0. Parameter estimates also gave little support for yearly variation in cost (Supporting Information).

We then tested the all-else-equal assumption that the mean number of surviving offspring is equal for asexual and sexual females (i.e., female fecundity-survival ratio, r , is 1). Based upon our estimates of the cost of sex c and the primary sex ratio s of *P. antipodarum* (Supporting Information), we found support for the all-else-equal assumption of Maynard Smith's model. For s equal to 0.5, our estimate of the fecundity-survival ratio r is 1 for model 2 ($c = 2$) and 1.12 (95% CI [0.91, 1.35]) for model 3, consistent with the all-else-equal assumption. We also calculated r assuming that the primary sex ratio is equal to the secondary sex ratio observed in the experiment: $s = 0.61$ (Supporting Information). Our estimate of r is then 1.22 for model 2 and 1.36, [1.11,

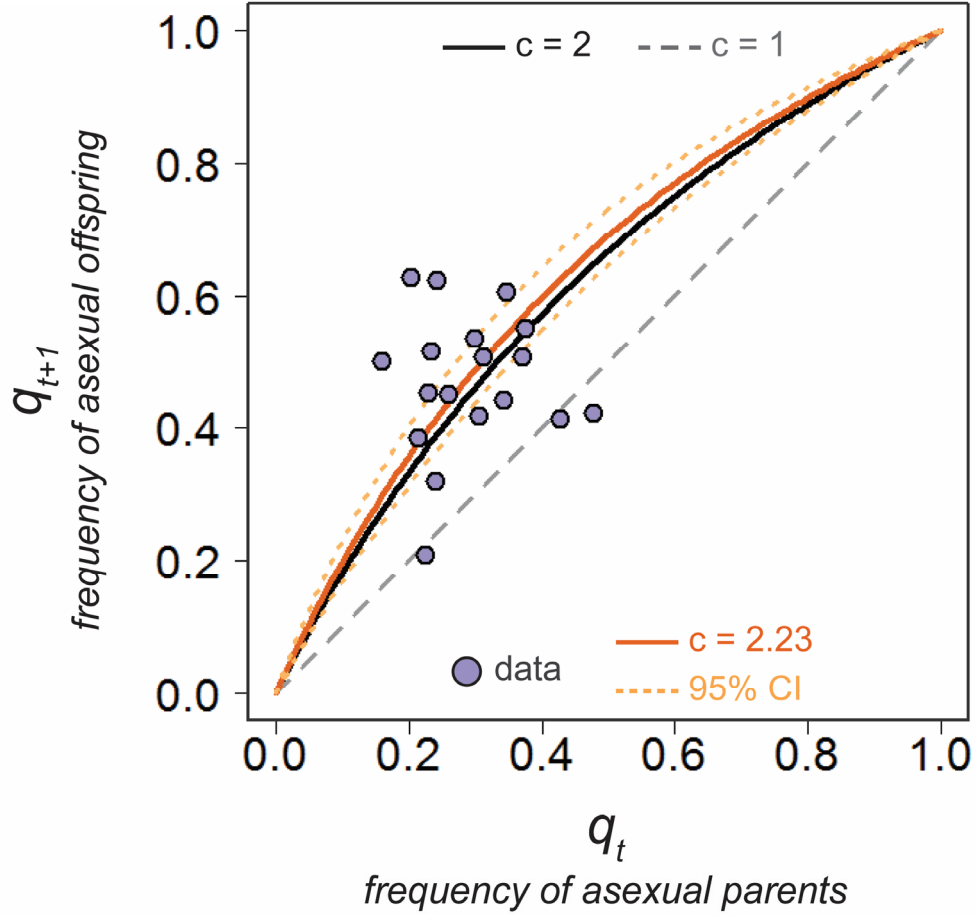


Figure 3: Experimental data are consistent with model predictions of a two-fold cost of sex. We fit our theoretical formulation of the cost of sex (Fig. 1c; equation (4)) to experimental data (Fig. 2b) on the frequency of asexuals q in generations t and $t+1$ in semi-natural mesocosms (purple dots). We used standard maximum likelihood techniques and Akaike's information criterion to compare different estimates of the cost of sex c in *P. antipodarum*. The predicted frequency of asexual offspring (q_{t+1}) for a given frequency of asexual parents (q_t) is shown for three values of the cost of sex: no cost ($c=1$, gray dashed line), a two-fold cost ($c=2$, black solid line), and the maximum likelihood estimate ($c=2.23$, solid orange line). The 95% confidence intervals of the maximum likelihood estimate include two ($c = [1.82, 2.69]$, dotted orange lines). Each point represents one mesocosm. For each mesocosm, the average number of triploid parents was 21.89 ± 1.63 SEM and the average number of triploid offspring was 31.67 ± 2.04 .

1.64] for model 3. Estimates of r above 1 are consistent with our finding that, in the mesocosms, the brood pouches of asexual females contained 19% more embryos on average than those of sexual females (Supporting Information). We conclude that asexual females produce an equivalent, or perhaps slightly greater, number of viable offspring than sexual females. Clearly, reduced fitness does not accompany the transition to asexuality and elevated ploidy in *P. antipodarum*.

Asexual lineages should therefore rapidly outcompete sexual lineages. Why then do sexual and asexual *P. antipodarum* coexist in nature? Sexual individuals comprised $70.8 \pm 2.0\%$ SEM of our field-collected juveniles from 2012-2014, in spite of their (at least) two-fold disadvantage. The natural ecology of *P. antipodarum* could reduce, or counterbalance, the cost of sex in the field. First, the extent to which the full cost of sex is realized depends upon death rate (Doncaster et al. 2000; Lively 2010b) and a population's proximity to carrying capacity (Doncaster et al. 2000; Lively 2011). In natural populations of *P. antipodarum*, death rates may be slightly lower and population sizes closer to carrying capacity than in our experimental populations. Theoretical models predict that the full two-fold cost of sex might not be realized in the field. Second, previous studies have suggested that coevolving parasites have the potential to counterbalance the cost of males, enabling coexistence (Vergara et al. 2014). Long-term field studies (Jokela et al. 2009) and a laboratory experiment (Koskella and Lively 2009) show that common *P. antipodarum* clones decline in frequency over time as they become disproportionately infected by the sterilizing trematode *Microphallus*. Thus sexual snail lineages might be maintained by parasite-mediated selection against common clonal genotypes.

The long-term maintenance of sex is one of the core anomalies in evolutionary biology, and the two-fold cost of sex is the foundational assumption of the paradox. Here we have provided a general model and a straightforward way to measure the cost of sex in semi-natural, outdoor mesocosms. Our results provide a quantitative validation of the two-fold cost in a natural system, thereby justifying the search for short-term advantages to cross-fertilization.

Methods

Semi-natural mesocosms

In January of 2012, 2013, and 2014, juvenile *Potamopyrgus antipodarum* were collected by passing a net through *Isoetes kirkii* vegetation (~1 meter depth) at four sites along the southwestern coast of Lake Alexandrina (Fig. S1a). The sampled sites were 1st Fence, Swamp, 2nd Fence and West Point. These sites have been well-studied since 1994 (Jokela et al. 2009). We therefore knew that large numbers of snails could be found there and that both reproductive modes would be represented in collections taken from a depth of 1 meter.

We transferred all field samples to the University of Canterbury's Edward Percival Field Station in Kaikoura, NZ and sieved them with a 1.7 mm sieve to obtain juvenile snails, which are less than ~2.5 mm in length. We used juvenile snails to establish mesocosms, because we aimed to minimize selection by coevolving parasites. Juveniles have relatively low rates of infection with sterilizing trematodes (Levri and

Lively 1996). Each experimental replicate was initiated with a random sample of 200 snails from each site (total=800), so that each mesocosm was representative of the whole region of the lake. We transferred experimental replicates to 1000 L Dolav box pallets, filled with ~800 L of water (Fig. S1b). These were located outside the field station in Kaikoura, NZ, so that experimental populations experienced natural seasonal variation in temperature, weather, and photoperiod. We covered the tanks with shade cloth and added small amounts of spirulina to them for ~2 weeks following establishment. The mesocosms were then left unattended from mid-February until early January of the next year. Under natural temperature conditions, this is sufficient time for juveniles to mature and reproduce, but insufficient time for their offspring to reproduce. Therefore, only two generations were present in the mesocosms at the end of the experimental year. We then emptied the experimental tanks and sieved the experimental populations at 1.4 mm to separate offspring and parent snails into discrete generations.

Mesocosm data collection

For each mesocosm, a random sample of 150 parental snails (>1.4 mm, originally juveniles) were immediately dissected under a microscope to determine shell length in millimeters, sex, brooding status and brood size, and infection status. Infection frequency with sterilizing trematodes was $10.05 \pm 0.92\%$ SEM. These individuals were infected with trematodes prior to collection from the field; no parasite transmission occurred in the mesocosms because the trematodes' alternate hosts were not available. We excluded infected individuals from subsequent statistical analyses in an attempt to remove obvious

forces that may alter the relative fitness of sexual and asexual females beyond intrinsic differences in birth rates. The heads of all dissected females were individually frozen, shipped to Indiana University (IN, USA), and stored at -80°C until flow cytometry analysis to determine reproductive mode. Males were assumed to be sexual diploids (Neiman et al. 2011). We estimated the number of offspring in some mesocosms to be as high as 18,000 (data not shown), so we retained only a randomly sampled subset of the offspring (>200) from each mesocosm. This subset was maintained at the Edward Percival Field Station for ~5 weeks, with regular water changes and spirulina feedings, prior to being transported alive to Indiana University. At Indiana University, the offspring were promptly frozen and stored at -80°C until flow cytometry analysis. We analyzed both male and female offspring by flow cytometry because they were too young to be sexed.

Flow cytometry was conducted as outlined in the supporting information of Gibson et al. (2016c). Briefly, we bound the DNA of nuclei from homogenized snail heads (parents) or whole bodies (offspring) with the fluorescent dye propidium iodide (Sigma-Aldrich; St. Louis, MO, USA). We analyzed all samples at the Indiana University-Bloomington Flow Cytometry Core Facility on an LSRII flow cytometer (BD Biosciences; Franklin Lakes, NJ, USA) with the High Throughput Sampler Option and BD Biosciences FACSDiva software v6.1.3. Triploid asexual females can be differentiated from diploid sexual females because their ~50% larger genome size is detected as elevated fluorescence of triploid relative to diploid nuclei. We analyzed 3000 nuclei per sample for parents and 2000 nuclei per sample for offspring. For the parental generation, we analyzed 65 ± 5.36 SEM randomly sampled females per mesocosm, for a

total of 1,170. Of these, $4.23 \pm 1.35\%$ were excluded because of ambiguous DNA content. For the offspring generation, we analyzed 70.5 ± 2.18 randomly sampled snails per mesocosm, for a total of 1,269. Of these, $4.76 \pm 0.07\%$ were excluded. Samples were excluded if there were fewer than 1000 nuclei obtained for a parental snail or fewer than 400 for an offspring snail, if there was no discernible peak in fluorescence or multiple peaks, or if the peak fell between the gates that designate regions consistent with diploid vs. triploid nuclei. The majority of flow cytometry runs were conducted blindly: the person performing flow cytometry did not know the identity of the population from which a given sample was drawn.

Statistical analysis of mesocosm data

To determine if the frequency of asexual individuals increased from the parent to offspring generation, we fit a logistic model with the number of triploid (female) and diploid (male and female) individuals in a replicate generation as the binomial response variable (logit link function). Generation (parent, offspring), year (2012, 2013, 2014), and their interaction were categorical predictor variables. This is an appropriate test given the binomial nature of our data, and the structure of the response variable accounts for non-independence of snails from the same replicate generation. The test included 36 replicate generations (18 parent and 18 offspring), and sample sizes per replicate generation are reported in Figure 2. We initially fit this model as a generalized linear mixed model (function `glmer` in package `lme4`, R) with the experimental replicate as a random effect to account for non-independence of parent and offspring snails derived from the same

experimental population. The estimate of the variance of this random effect was zero. Therefore, we fit a simpler generalized linear model (function `glm` in R (R Core Team 2013)). We tested the significance of the each effect using a likelihood ratio test of models with and without the effect of interest. We calculated the fold-increase and 95% confidence intervals using the odds ratios for generation from these logistic models, the profile likelihood confidence intervals for the odds ratios, and the mean frequency of asexual individuals in the parental generation.

For the parental generation, we obtained the number of triploid females and the relative frequency of triploid vs. diploid females from the flow cytometry results. Because flow cytometry was only performed on a subset of females, we used the overall frequency of males and the relative frequency of triploid vs. diploid females in a replicate to estimate the total sample size that would have generated the observed number of triploid females. From this, we calculated the corresponding number of diploid (male and female) individuals for a replicate. For the offspring generation, flow cytometry was performed on male and female snails, so we obtained the overall number of triploid and diploid individuals directly from the flow cytometry results.

We tested if the binomial distribution was appropriate for our data using the variance inflation factor (\hat{c}), which is calculated as the Pearson goodness-of-fit test of model predicted values relative to the model's residual degrees of freedom. Values for the variance inflation factor that exceed 1 indicate overdispersion (Cox and Snell 1989). We found evidence that the data were slightly overdispersed with the binomial distribution ($\hat{c}=1.89$), indicating that the variance in our data exceeded that predicted by the binomial distribution. One way to correct for overdispersion is to correct the

estimated variance of model estimates by re-fitting the model with a quasi-binomial distribution (Crawley 2013). The quasi-binomial distribution can affect and confidence intervals and estimates of significance (Burnham and Anderson 1998). In the supplementary information, we report the significance of the generation effect, plus estimated model-predicted fold-increases in asexual frequency and confidence intervals under the quasi-binomial distribution. The results are qualitatively identical to those with the binomial distribution. We therefore report the binomial results in the main text for ease of interpretation.

Details of model selection

We assumed a beta-binomial distribution (package `emdbook` (Bolker 2008), R) for our likelihood function (details below). Our four candidate models (Table 1) specified different probabilities of observing the number of triploid offspring in the total number of offspring analyzed, given the frequency of triploid individuals in the parental generation. Using the `mle2` function (package `bbmle`, R) to find maximum likelihood estimates of parameters, we obtained the likelihood of each model given our experimental data, i.e. the product of the probabilities of each of the 18 mesocosm observations (18 paired estimates of q_t and q_{t+1}).

We compared models using Akaike's information criterion (Akaike 1973), corrected for small sample size (Sugiura 1978; Hurvich and Tsai 1991) (AIC_c : appropriate when the ratio of data points to parameters is less than 40) (Burnham and Anderson 1998):

$$AIC = -2 \log L + 2K$$

$$AIC_c = AIC + \frac{2K(K-1)}{n-K-1}$$

where K is the number of parameters and n is the number of data points ($n=18$). Better models have lower AIC, and adding parameters increases the value. The relative values of AIC, rather than the absolute values, are informative, so we calculated:

$$\Delta AIC_i = AIC_i - AIC_{min}$$

for each model i . AIC_{min} is the lowest AIC in the set of candidate models, so the best model has a ΔAIC of 0. Roughly, models with ΔAIC values below 2 have substantial support, models with ΔAIC from 4-7 have considerably less support, and models with ΔAIC above 10 have no support (Burnham and Anderson 1998).

Because these cut-offs are only rules of thumb, Burnham and Anderson (1998: pg. 128-129) recommend estimating the sampling distribution of ΔAIC using bootstrapping in order to calculate the ΔAIC value that delineates a 95% confidence set of models. We followed their recommended procedure by bootstrapping our data set 10,000 times with replacement. For each bootstrapped data set, we fit our four candidate models and calculated ΔAIC for model 2 (our best model) by subtracting the minimum AIC value in each bootstrap replicate from the AIC value for model 2 in that replicate. We identified the value of ΔAIC_2 that was greater than or equal to 95% of the ΔAIC_2 values obtained in the bootstrapping analysis. The confidence set of models is defined as those having ΔAIC less than or equal to this limit in the actual data analysis.

In addition, we calculated Akaike weights, w , which can be interpreted as the weight of evidence for a model, or the probability that model i is the best model amongst the set of R candidate models (Akaike 1978; Burnham and Anderson 1998):

$$w_i = \frac{\exp\left(-\frac{1}{2}\Delta_i\right)}{\sum_{r=1}^R \exp\left(-\frac{1}{2}\Delta_r\right)}$$

Lastly, we bootstrapped our data set 10,000 times with replacement and re-ran model fitting to estimate 95% confidence intervals for parameter estimates.

Justification of beta-binomial distribution for likelihood functions

When we initially assumed a binomial distribution for the likelihood functions, we found that the variance inflation factor for our global model (model 4) was substantially greater than 1 ($\hat{c}=4.80$). A variance inflation factor of this magnitude is consistent with severe overdispersion and indicates that the binomial distribution is not appropriate for our data. We therefore assumed a beta-binomial distribution, which better models variation in data by estimating an additional parameter θ to allow for variation in the per-trial probability (Crowder 1978; Bolker 2008). Small estimates of θ are consistent with larger overdispersion (Bolker 2008). A likelihood ratio test comparing the global model with a binomial vs. beta-binomial distribution strongly justified use of the beta-binomial ($\chi^2 = 42.42$, $df = 1$, $p < 0.001$). Similarly, the beta-binomial model had a lower AIC_c , and ΔAIC_c was very large (39.06). Consistent with these results, the maximum likelihood estimate of θ was small (17.71) (global model, beta-binomial distribution).

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Supporting Information

Life-history comparisons: field-collected adults

To test the all-else-equal assumption for fecundity, we re-assessed the life-history comparisons of Jokela et al. (1997b) for our study population in 2013 and 2014. We collected snails from our study sites at Lake Alexandrina as described in the Methods. When field samples were sieved at 1.7 mm to obtain experimental juvenile snails, we reserved the adult snails (>1.7 mm) for life-history comparison. For 150 individuals per

site, we determined shell length in millimeters, sex, brooding status, and infection status. For the first 20 females identified as brooding, we counted the number of eggs in the brood. We froze the heads of dissected females and determined reproductive mode for 50 females per site per year using flow cytometry, as outlined in the Methods.

To test if age at reproductive maturity varied with reproductive mode, we fit a linear model with reproductive mode, year, and site as predictors of the length of individual brooding females (Gaussian distribution, identity link function). To test if the probability of brooding varied with reproductive mode, we fit a generalized linear model with brooding status (yes, no) of individual uninfected females as a binomial response variable (logit link function). Females infected with sterilizing trematodes were excluded. Reproductive mode, year and site were categorical factors, and length (proxy for age) was included as a covariate. To test if brood size (a proxy for fecundity) varied with reproductive mode, we fit a generalized linear model with brood size (negative binomial, log link function) of individual brooding females as the response variable. Predictor variables were identical to those in analyses of brooding probability. For each model, we tested the significance of reproductive mode using a Wald test. Interactions were tested in each analysis. They were all consistently insignificant and thus excluded. Analyses were performed in SPSS v23 (IBM). We verified that the assumptions of the different models were met.

Length of brooding females, a proxy for age at maturity, varied with reproductive mode ($n=70$; Wald $\chi^2 = 4.27$, $df=1$, $p=0.04$), with asexual females brooding at 96% the length of sexual females, on average (estimated marginal means: asexual – 4.61 mm, 95% CI [4.49, 4.73] vs. sexual – 4.79 [4.66, 4.93]). Uninfected asexual females were

three times more likely to be brooding than uninfected sexual females ($n=301$; Wald $\chi^2 = 7.32$, $df=1$, $p=0.007$; estimated marginal means: asexual – 0.12, 95% CI [0.07, 0.22] vs. sexual – 0.04 [0.02, 0.09]). These brooding asexual females carried 61% fewer embryos in their brood pouches than did sexual females, though this difference was marginally significant ($n=70$; Wald $\chi^2 = 3.45$, $df=1$, $p=0.063$; estimated marginal means: asexual – 11.71, 95% CI [8.11, 16.91] vs. sexual – 19.31 [13.17, 28.31]). As a whole, these life-history comparisons show no evidence for a reduction in fecundity associated with the transition to asexual reproduction. In fact, the clonal lineages currently in Lake Alexandrina may have an advantage over sexual females given their significantly elevated rates of brooding.

Life-history comparisons: mesocosm parents

We also compared life-history traits of female parents in our mesocosms to determine if violations of the all-else-equal assumption arose in the tank environment. The Methods section describes the collection of life-history data from parental snails. For all traits, we fit generalized estimating equations (GEE) with the experimental replicate as the subject variable with an exchangeable variance-covariance matrix. GEEs allow specification of the correlation between individuals derived from the same experimental replicate (Liang and Zeger 1986; Zeger and Liang 1986), and the exchangeable variance-covariance matrix indicates that the correlation between individuals from the same experimental replicate does not vary between replicates (Zuur et al. 2009). Models were otherwise specified as described for field-collected snails. For variation in length, predictor

variables were reproductive mode, year (2012, 2013, and 2014), and their interaction. For variation in the probability of brooding and in brood size, reproductive mode, year and their interaction were categorical factors, and length (proxy for age) was included as a covariate. GEEs were performed in SPSS. We verified that the assumptions of the different models were met.

Length of brooding females did not vary with reproductive mode ($n=701$, Wald $\chi^2 = 2.80$, $df=1$, $p=0.09$; estimated marginal means: asexual – 4.86 mm, 95% CI [4.78, 4.95] vs. sexual – 4.79 [4.75, 4.83]). Based upon estimated marginal means and 95% confidence intervals for shell length, sexual females in the mesocosms reached reproductive maturity at the same size as females in the field, while asexual females reached reproductive maturity at a slightly larger size in the mesocosms vs. the field. The probability of brooding did not vary with reproductive mode ($n=999$, Wald $\chi^2 = 1.41$, $df=1$, $p=0.234$; estimated marginal means: asexual – 0.79, 95% CI [0.65, 0.88] vs. sexual – 0.74 [0.50, 0.89]). Both sexual and asexual females were much more likely to be brooding in the mesocosms than in the field collections. Brooding asexual females carried 19% more embryos in their brood pouches than did sexual females ($n=701$; Wald $\chi^2 = 13.46$, $df=1$, $p<0.001$; estimated marginal means: asexual – 9.10, 95% CI [6.93, 11.96] vs. sexual – 7.66 [6.26, 9.38]). The direction of this relationship differs from that observed in the field. Comparing estimated marginal means and 95% confidence intervals, brood sizes for sexual females were far lower in the mesocosms than in the field, while brood sizes were similar for asexual females across samples. As for the life-history comparisons with the field collections, asexual females in the mesocosms show

no reduction in fecundity relative to sexual females. Rather, larger brood sizes may give asexual females an advantage, consistent with our finding for field-collected females.

Secondary sex ratio in field-collected parents and mesocosm adults

As we show in equation (7), the cost of sex c can be written as a function of r , the female fecundity-survival ratio and s , the frequency of females in the sexual population. More specifically, equation (7) says that the cost of sex is an inverse function of the proportion of resources allocated by sexual mothers to daughters vs. sons. Assuming that sons and daughters are equally costly (Fisher 1930), we represent this proportion as s , the primary sex ratio of broods of sexual females.

We do not know the primary sex ratio of sexual *P. antipodarum* at Lake Alexandrina. It is difficult to determine because of the many factors that can distort the original sex ratio of a brood (Yusa 2007). Our *a priori* prediction is a sex ratio of 50% female. Populations of *P. antipodarum* are very large (Hamilton 1967; Paczesniak et al. 2014). In addition, some other prosobranch snails have chromosomal sex determination with females heterogametic (Baršienė et al. 2000; Yusa 2007). The mechanism of sex determination is not, however, characterized for *P. antipodarum* (Wallace 1992; Neiman et al. 2012).

To estimate a range for the primary sex ratio, we investigated the secondary or apparent sex ratio, which we calculated as the frequency of sexual females in the sexual subpopulations of our study populations. For mesocosm parents, we fit a binomial distribution to the number of diploid female and male snails in our 18 experimental

replicates and used the `mle2` function (package `bbmle`, R) to find the maximum likelihood estimate of the probability that a diploid snail is female (sex ratio). We used the function `confint` to obtain 95% confidence intervals on the estimate. We did the same for field-collected adults, fitting a binomial distribution to the number of diploid female and male snails at each of our four sites in 2013 and 2014. We used a likelihood ratio test to compare these models against models in which the binomial probability was fixed at 0.5.

For mesocosm parents, the maximum likelihood estimate of the secondary sex ratio was 61% female (95% CI [0.59, 0.64]). For field-collected adults, the maximum likelihood estimate of the secondary sex ratio was 69% female (95% CI [0.65, 0.72]). For both samples, these models fit our data substantially better than a model with the sex ratio fixed at 50% female (likelihood ratio test: mesocosms - $D = 99.82$, $df = 1$, $p < 0.001$; field - $D = 113.47$, $df = 1$, $p < 0.001$). The secondary sex ratio was more female-biased in the field's sexual population than in the mesocosms'. Early male mortality may explain this difference and could generally contribute to the female bias in secondary sex ratios. Based upon these results, we concluded that the primary sex ratio likely lies between 50% and 61% female ($0.50 \leq s \leq 0.61$).

Overdispersion and the quasi-binomial

For the logistic model used to evaluate differences in the frequency of asexual individuals in parent vs. offspring generations, the variance inflation factor exceeded 1 ($\hat{c} = 1.89$), indicating overdispersion under the binomial distribution. A standard correction for overdispersion is to re-fit the model with a quasibinomial distribution (Crawley 2013),

which corrects the estimated variance of coefficients. Here we compare the results of the binomial model reported in the main text with those of a quasi-binomial model.

Comparisons using likelihood ratios are not possible with the quasi-binomial, so we compare estimates of the overall contribution of each predictor using Wald tests. Use of the quasi-binomial does not alter estimates of coefficients, but it can increase the variance of those estimates.

Use of the quasi-binomial distribution did not qualitatively alter our conclusions. There was an overall effect of generation (binomial: Wald $\chi^2 = 25.5$, $df=1$, $p<0.001$; quasi-binomial: Wald $\chi^2 = 13.5$, $df=1$, $p<0.001$), and the frequency of asexual individuals increased substantially from parent to offspring generations (binomial: 1.56, 95% CI [1.33, 1.79]); quasi-binomial: 1.56, [1.25, 1.88]). There was also an effect of year (binomial: Wald $\chi^2 = 16.4$, $df=2$, $p<0.001$; quasi-binomial: Wald $\chi^2 = 8.7$, $df=2$, $p=0.013$), with the overall frequency of asexuals higher in the latter two years of replication (binomial: odds ratio vs. 2012: 2013= 1.73 [1.31, 2.31], 2014 = 1.61 [1.21, 2.15]; quasi-binomial: odds ratio vs. 2012: 2013= 1.73 [1.18,2.57], 2014 = 1.61 [1.09,2.38]). There was no overall effect of the interaction of generation and year (binomial: Wald $\chi^2 = 1.7$, $df=2$, $p=0.440$; quasi-binomial: Wald $\chi^2 = 0.87$, $df=1$, $p=0.65$).

Yearly variation in the cost of males

As discussed in the main text, there was weak support for model 4, in which the magnitude of the cost of sex varies between the three years in which the experiment was replicated ($\Delta AIC_c = 6.87$, $w = 0.02$). Parameter estimates from model 4 are consistent

with this finding. The maximum likelihood estimate of the baseline cost in 2012 was slightly below two ($c_0 = 1.94$, 95% CI [1.32, 2.61]). The maximum likelihood estimate for the difference from this 2012 baseline included zero for both 2013 ($d_2 = 0.15$, [-0.66, 0.99]) and 2014 ($d_c = 0.82$, [-0.05, 1.79]), indicating no significant difference from 2012 in either year.

Table S1: Numbers of asexual females (triploid) and sexual females and males (diploids) in parent and offspring generations of experimental mesocosms. Flow cytometry directly provided the number of asexual individuals for both parents and offspring and the number of sexual individuals for offspring. The number of sexual parents is derived from the frequency of males and the relative frequency of diploid and triploid females in a mesocosm.

Year	Replicate	Parents			Offspring		
		N asexual	N sexual	Frequency asexual	N asexual	N sexual	Frequency asexual
2012	1	20	46	0.304	23	32	0.418
2012	2	30	99	0.233	30	28	0.517
2012	3	30	86	0.259	37	45	0.451
2012	4	30	110	0.214	22	35	0.386
2012	5	29	92	0.240	18	38	0.321
2012	6	29	100	0.226	12	45	0.211
2013	1	20	44	0.313	37	36	0.507
2013	2	14	44	0.243	43	26	0.623
2013	3	18	35	0.341	31	39	0.443
2013	4	23	38	0.375	38	31	0.551
2013	5	23	43	0.347	43	28	0.606
2013	6	28	31	0.478	30	41	0.423
2014	1	20	34	0.371	35	34	0.507
2014	2	12	40	0.229	29	35	0.453
2014	3	27	36	0.427	29	41	0.414
2014	4	20	47	0.299	37	32	0.536
2014	5	11	43	0.204	42	25	0.627
2014	6	10	52	0.160	34	34	0.500

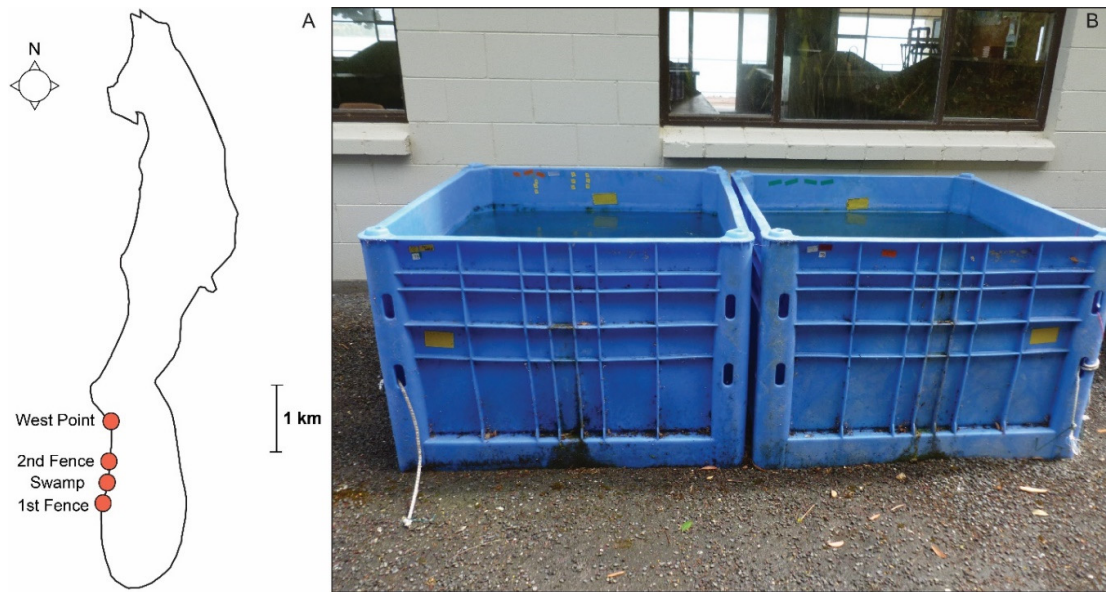
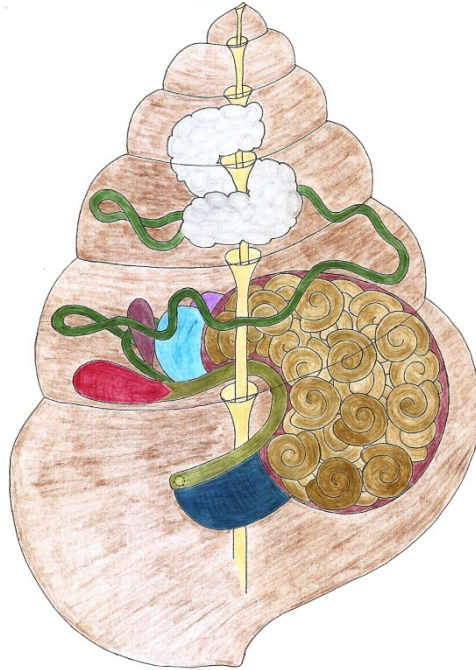
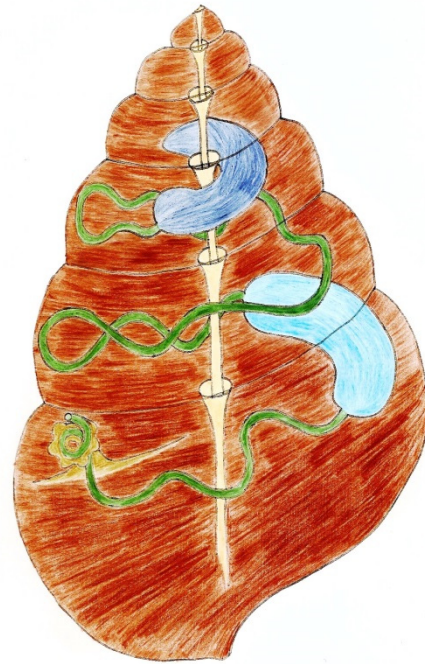


Figure S1: Establishment of experimental mesocosms. (A) Juvenile snails were collected at ~1 meter depth from four well-studied sites along the southwestern coast of Lake Alexandrina (Mackenzie Basin, South Island, New Zealand). (B) 800 juveniles, 200 from each site, were added to 800-liter Dolav bins outside the University of Canterbury's Edward Percival Field Station in Kaikoura, New Zealand. The tanks are shown uncovered one year after the start of an experimental run. They are covered with dark shade-cloth during the year.

Female reproductive tract



Male reproductive tract



Reproductive tracts of female (left) and male (right) *Potamopyrgus antipodarum*. Female structures are: embryos (brown) in distended brood pouch (crimson), oviduct (green), ovary (white), mucus gland (light purple), albumen gland (light blue), receptaculum seminis (dark purple), bursa copulatrix (red), ventral channel/sperm groove (olive green) plus genital opening (yellow dot), capsule gland (dark blue), and columella (yellow column). Male structures are: prostate gland (light blue), testes (dark blue), vas deferens (green) ending in dextral opening of penis (tan), and columella (yellow column). Used with permission of artist Amanda Nelson.

“The ‘cost of sex’ has proven to be a slippery concept.”

~ Curtis M. Lively, *Lectures on Mathematics in the Life Sciences* (1990), 22: 93-107.

Chapter 2

Fine-scale spatial covariation between infection prevalence and
susceptibility in a natural population

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*“I want to suggest that the struggle against disease, and particularly
infectious disease, has been a very important evolutionary agent, and that
some of its results have been rather unlike those of the struggle against
natural forces, hunger, and predators...”*

~ J.B.S. Haldane, *La Ricerca Scientifica* (1949), 19: 68-76.

Abstract

The prevalence of infection varies dramatically on a fine spatial scale. Many evolutionary hypotheses are founded upon the assumption that this variation is due to host genetics, such that sites with a high frequency of alleles conferring susceptibility are associated with higher infection prevalence. This assumption is largely untested and may be compromised at finer spatial scales where gene flow between sites is high. We put this assumption to the test in a natural snail-trematode interaction in which host susceptibility is known to have a strong genetic basis. A decade of field sampling revealed substantial spatial variation in infection prevalence between 13 sites around a small lake. Laboratory assays replicated over three years demonstrate striking variation in host susceptibility among sites, in spite of high levels of gene flow between sites. We find that mean susceptibility can explain over one-third of the observed variation in mean infection prevalence among sites. We estimate that variation in susceptibility and exposure together can explain the majority of variation in prevalence. Overall, our findings in this natural host-parasite system argue that spatial variation in infection prevalence covaries strongly with variation in the distribution of genetically based susceptibility, even at a fine spatial scale.

Introduction

The prevalence of infection varies in space. This heterogeneity is evident on a very fine scale for a wide range of host-parasite systems, including dengue virus (Yoon et al. 2012), anther smut (Burdon and Thrall 1999: figure 2), cholera (Snow 1855), powdery

mildew (Laine 2006), and human schistosomiasis (Rudge et al. 2008). The difference between nearby sites can be dramatic. For example, Woolhouse and Chandiwana (1989) measured infection prevalence of the freshwater snail *Bulinus globosus* with the trematode agent of human schistosomiasis. At 22 sites along an 860 meter stretch of river, they discovered that infection prevalence ranged from 0 to 60%. Their study exemplifies one of the motivations for documenting and explaining heterogeneity in infection prevalence: the density of snails and their frequency of infection at a given site determine a human individual's risk of contracting schistosomiasis (Woolhouse and Chandiwana 1990; Clennon et al. 2006).

Why does the prevalence of infection vary so much on small spatial scales? We are gaining a greater understanding of how ecological factors influence variation in infection prevalence. These factors include habitat structure (Grosholz 1993; Grosholz and Ruiz 1995; Penczykowski et al. 2014), competition (Grosholz 1992; Hall et al. 2009), enrichment and land use (Johnson et al. 2007; McKenzie 2007; King et al. 2010), resources (Duffy et al. 2012; Satterfield et al. 2015), temperature and climate (Linthicum et al. 1999; Stapp et al. 2004; Bruno et al. 2007), and the presence of roads (Altman and Byers 2014; Jousimo et al. 2014).

In contrast, we know much less about how infection prevalence is affected by genetic variation for susceptibility (as reviewed in Little 2002). A core assumption of many evolutionary hypotheses is that hosts evolve in response to parasite-mediated selection (e.g. maintenance of polymorphism at infection loci and the Red Queen - Haldane 1949; Jaenike 1978; Hamilton 1980; Hedrick 1994; Apanius et al. 1997; Dangel and Jones 2001; Hughes 2002; Watson et al. 2005), which requires standing genetic

variation for resistance/susceptibility. However, variation due to environmental factors might overwhelm any contribution of genetic variation to variation in infection prevalence, weakening the response to parasite-mediated selection.

Studies in a few plant-fungal systems have documented extensive genetic variation in host susceptibility (as reviewed in Laine et al. 2011) and connected that variation in susceptibility to variation in infection prevalence within and between natural populations (Thrall and Jarosz 1994a; Thrall and Jarosz 1994b; Alexander and Antonovics 1995; Thrall and Burdon 2000; Thrall et al. 2001; Laine 2004; Laine 2006). For example, Jousimo et al. (2014) recently demonstrated that powdery mildew was less likely to establish and persist in more resistant populations of its host, the ribwort plantain.

Similar studies are rare in animal systems, and results are contradictory. We might predict that the potential for significant mobility of animal hosts may reduce the contribution of genetics to spatial variation in infection prevalence. Indeed, Grosholz and Ruiz (1995) argued that high gene flow erodes genetic variation for susceptibility of xanthid crabs to castrating *Sacculina* barnacles. Though barnacle prevalence varied markedly between crab populations, they found no evidence of genetic variation in susceptibility. Even if susceptibility does vary, it may have little explanatory power: Scott (1991) found that laboratory mouse strains did not differ in nematode prevalence in semi-natural mesocosms, even though they differed significantly in susceptibility. In contrast, studies of *Daphnia* (Little and Ebert 2000) and a wild sheep population (Hayward et al. 2014; Nussey et al. 2014) link estimates of increased genetic susceptibility to increased infection prevalence and mortality, respectively, in the field. Additional work on *Daphnia*

demonstrates within-population variation in susceptibility to a fungal parasite (Duffy et al. 2008) and parasite-mediated selection on susceptibility (Duffy et al. 2008; Duffy et al. 2012) that corresponds to a termination of epidemics (Duffy et al. 2009).

These mixed findings call into question the degree to which infection prevalence covaries with susceptibility at a fine spatial scale. By “fine,” we mean the scale at which gene flow between sites is expected to be high (Richardson et al. 2014). Snail-trematode interactions provide a promising avenue in which to develop this line of research.

Infection prevalence is highly variable (Pesigan et al. 1958 pg. 568-571; Robson and Williams 1970; Anderson and May 1979; Curtis and Hurd 1983; Woolhouse and Chandiwana 1989; Jokela and Lively 1995b; Jokela et al. 1997a; Smith 2001; Vergara et al. 2013), and genetic variation explains a great deal of variation in susceptibility (Newton 1953; Richards and Merritt 1972; Richards 1975; Basch 1976; Wakelin 1978; Webster and Woolhouse 1998; Negovetic and Jokela 2001). This is the case in our system, the freshwater snail *Potamopyrgus antipodarum* and its sterilizing trematode *Microphallus* sp. Laboratory assays consistently demonstrate that the relative susceptibility of hosts has a strong genetic basis (Dybdahl and Krist 2004; Krist et al. 2004; Jokela et al. 2009) that arises largely from the interaction of host and parasite genotypes (Lively 1989; Lively et al. 2004). Thus any covariance of infection prevalence and susceptibility would likely reflect the contribution of genetic variation to variation in prevalence.

Here, we quantify fine-scale spatial variation in infection prevalence and susceptibility in this natural snail-trematode interaction. We then test the hypothesis that infection prevalence covaries with susceptibility. We conducted our study at a small New

Zealand lake (6.4 km²), where gene flow between shoreline sites is sufficiently high that hosts and parasites from distant sites are not differentiated at neutral loci (Dybdahl and Lively 1996; Fox et al. 1996; Paczesniak et al. 2014). Ten years of field data demonstrate striking spatial and temporal variation in infection prevalence at 13 shoreline sites. Experimental inoculations of hosts from these sites reveal a similar degree of spatial variation in susceptibility. We found that mean susceptibility can explain over one-third of the variation in mean field prevalence, with more susceptible sites having higher prevalence. Overall, our results strongly argue that genetic variation for susceptibility between sites persists in the face of high gene flow and makes a substantial contribution to fine-scale spatial variation in infection prevalence.

Methods

Natural history

Potamopyrgus antipodarum is a prosobranch gastropod that is abundant in New Zealand lakes and streams. It is the first intermediate host to at least twenty species of trematode parasites (Hechinger 2012). The best studied of these is *Microphallus* sp., which is highly virulent, sterilizing both male and females snails. Snails ingest parasite eggs while foraging and become sterilized as the developing infections replace the host gonads with larval metacercariae. The definitive host (ducks) ingests the infected snail while foraging, and *Microphallus* matures and sexually reproduces in the duck intestine. The parasite releases its eggs in the duck's feces, which are then dispersed in the environment (Hechinger 2012). We will hereafter use "host" to refer to the intermediate snail host.

We conducted this study at Lake Alexandrina, a 6.4 km² lake in the Mackenzie Basin of the central South Island of New Zealand. *Microphallus* is prevalent at this lake, with infection frequencies exceeding 60% at some sites (Jokela et al. 2009). Lake Alexandrina is home to several resident bird populations, including *Anas platyrhynchos* (introduced Mallards), *A. superciliosa* (native Grey ducks), their hybrids, and *Aythya novaeseelandiae* (New Zealand scaup), the major definitive hosts of *Microphallus* sp. (Osnas and Lively 2011). Female *P. antipodarum* vary in reproductive mode at Lake Alexandrina, with asexual females coexisting with sexual males and females (Winterbourn 1970; Jokela et al. 2009). In this study, we investigate overall patterns across sexual and asexual lineages combined.

We focus upon the lake's shallow, shoreline habitat (<0.5 m deep). *Microphallus* prevalence in snails is higher here than in deeper habitats (Jokela and Lively 1995b; Jokela and Lively 1995a). The shoreline is easily accessible along the lake's southern half, making it ideal for investigation of fine-scale spatial patterns. Consistent with *P. antipodarum*'s potential for passive dispersal (Hubendick 1950; Ribi 1986), gene flow between shoreline sites occurs readily, as evidenced by a lack of genetic structure at neutral loci (Fox et al. 1996; Paczesniak et al. 2014). There is also no structure at neutral loci for *Microphallus* (Dybdahl and Lively 1996), as we would predict for a parasite dispersed by mobile waterfowl.

Does infection prevalence vary in space?

We used a long-term data set to examine spatial variation in infection prevalence at Lake Alexandrina. Each year from 2006 to 2015, we visited 12-13 shoreline sites in mid-January to February. We sampled a large number of individuals at each site by sweeping a net along the bank and through vegetation. These samples were transported to the University of Canterbury's Edward Percival Field Station (Kaikoura, New Zealand). We performed dissections at the field station or after transportation to ETH Zurich (Switzerland) or Indiana University (Indiana, USA). For each site, we randomly selected approximately 100 snails and determined length (mm), gender, and infection status. In 2014 and 2015, samples were sieved at >1.7 mm, excluding snails below ~ 3 mm.

We restricted analysis to female snails for consistency with susceptibility analyses (see following section). We tested the hypothesis that infection prevalence varies between sites and years using a generalized linear model with site, year, and their interaction as predictors of the probability of infection of an individual female (binomial with logit link function) (SPSS v21, IBM). Each female's shell length was included as a covariate to control for age-related variation in prevalence. The cumulative risk of infection increases with age, and shell length correlates positively with age within habitats (Jokela and Lively 1995b). We determined if the binomial distribution was appropriate for our data by testing for overdispersion. If a model's residual deviance is less than or approximately equal to the residual degrees of freedom, data are not overdispersed and the specified distribution of the model is appropriate (Crawley 2013). We found no evidence for overdispersion: the ratio of residual deviance to degrees of freedom was 1.

We used spatial analyses of infection prevalence to determine if sites were independent of one another in space. GPS coordinates and geographic distances between sites were obtained using Google Earth. For these analyses, we used “length-corrected” estimates of prevalence (estimated marginal means from generalized linear model, above) with an arcsine transformation. First, we tested the hypothesis that nearby sites have similar prevalence with a Mantel test in the *vegan* package v2.3-0 (Dixon and Palmer 2003)(defaults = Pearson correlation, 999 permutations) in R (R Core Team 2013). Specifically, we measured the correlation of straight-line geographic distance with the absolute value of the difference in mean infection prevalence (equivalent of Euclidean and Manhattan distances for our data). Secondly, we tested the hypothesis that mean prevalence shows spatial autocorrelation by estimating Moran’s I, and its significance, in the *ape* package v3.3 (Paradis et al. 2004) in R. Lastly, we tested the hypothesis that there is a geographic cline in prevalence using a generalized estimating equation (GEE) with latitude and longitude as predictors of prevalence (linear response variable) in SPSS. GEEs were developed for analysis of longitudinal data and can account for the correlation between measurements taken at the same site through time (Liang and Zeger 1986; Zeger and Liang 1986). Site was included as a subject variable and year (2006-2015) as a within-subject variable with a first-order autoregressive variance-covariance matrix. This is the preferred correlation structure for longitudinal data (Wang and Carey 2003; Ziegler and Vens 2010; Vens and Ziegler 2012). We also tested for a correlation of mean infection prevalence in recent years (2013-2015) with latitude and longitude (Spearman’s rank correlation due to deviations from normality).

Does susceptibility vary in space?

To evaluate the hypothesis that variation in prevalence arises from variation in host susceptibility, we first tested the prediction that susceptibility varies between sites. We performed artificial inoculations to measure the susceptibility of juvenile hosts from different sites around the lake. Juvenile snails are ideal for artificial inoculations, because they have experienced relatively little parasite exposure (Levri and Lively 1996) and are susceptible to infection (Krist and Lively 1998). We defined susceptibility as the infection rate obtained following standardized exposure to parasites in the lab. To ensure that infection rate in exposed replicates truly reflected susceptibility, we used high doses of parasites for inoculation. In doing so, our goal was to attain a saturating dose such that every host encountered a sufficiently large number of parasites to become infected if susceptible. It is possible that juvenile snails had acquired infection in the field prior to collection. To estimate the total frequency of susceptible snails at a site, we added to these field infections using artificial inoculations. In other words, the infection rate measured in exposed replicates (susceptibility) reflected both infections gained in the field and in artificial inoculations.

Our assay of susceptibility is not a direct measure of genetic variation in susceptibility: experimental snails were collected directly from the field, and thus susceptibility includes any variation due to maternal effects and early life experience. Prior studies, however, strongly argue that genetic variation explains the majority of variation in susceptibility: relative susceptibility of host genotypes is not affected by host condition (Dybdahl and Krist 2004), nor by the upregulation of plastic immune responses (Osnas and Lively 2005, 2006). Selection on host susceptibility results in a strong

response to selection, which requires genetic variation for susceptibility (Koskella et al. 2011). Moreover, the interaction of host and parasite genotype explains the majority of variation in susceptibility (Lively et al. 2004), and outbreeding depression of hybrid parasites is consistent with nonadditive gene effects and genotype specificity for susceptibility (Dybdahl et al. 2008).

In February of 2013, 2014, and 2015, we collected snails from shoreline sites (2013: n=12, Halfway excluded; 2014, 2015: n=13), as described above. We also collected duck feces from the lake shore to obtain parasite eggs that are infective to snails. In 2013 and 2015, we combined duck feces from multiple sites around the entire lake. In 2014, we made separate collections from a southern (Source 1) and northern (Source 2) site (Fig. 1A: starred sites). We transported all samples to the Edward Percival Field Station. We sieved snail collections at <1.7 mm to obtain juvenile snails. The duck feces were repeatedly rinsed with fresh water to remove contaminants, homogenized, and sieved to remove debris.

For each site, we established replicates of 100 (2013) or 75 (2014, 2015) snails each. Two replicates per site were not exposed to parasites (Control treatment). The Control treatment allowed us to measure variation in early-life exposure of juveniles from different sites. Control snails received a light feeding of spirulina in lieu of parasite exposure. For replicates in the Exposed treatment, homogenized duck feces were added to the water of the containers over the course of eight (2014, 2015) or 10 days (2013). In 2013 and 2015, four replicates were exposed to 1500 and 2900 eggs/snail, respectively. In 2014, three replicates were exposed to 800 eggs/snail of Source 1 and three replicates to 2100 eggs/snail of Source 2. We used a Neubauer hemocytometer to determine egg

concentrations. Such high doses are commonly used when exposing *P. antipodarum* to *Microphallus* (Osnas and Lively 2004; King et al. 2011a), and mortality in exposed replicates did not exceed that in control replicates (Appendix I). Doses used in different sources and years do vary substantially, but it is very likely that all doses were sufficiently high to exceed the minimum threshold required to infect all susceptible hosts (Osnas and Lively 2004).

We maintained our experimental replicates until 80 days post-exposure to allow for parasite development. We then dissected each snail to determine, length, gender, and infection status. The subsequent analyses are restricted to female snails. They are preferable for evaluating susceptibility in artificial inoculations, because males tend to be relatively rare at some sites (as little as 16% of juveniles) and vary significantly in size and behavior.

To evaluate susceptibility assays in 2013 and 2015, we used the function `glm` in R to fit generalized linear models with the number of infected and uninfected females in a replicate as a binomial response variable (logit link function). We first determined if artificial exposures increased the probability of infection by evaluating the treatment effect in a model with treatment, site, and their interaction as factors (years evaluated separately). We used the function `confint` to obtain confidence intervals for the odds ratios.

We found that artificial inoculations were successful (see Results), so we restricted analysis to the Exposed treatment to evaluate variation in susceptibility (infection frequency in exposed replicates). We fit generalized linear models with site as

a predictor of the number of infected and uninfected females in exposed replicates. We used a likelihood ratio test to test the significance of the site effect, relative to an intercept-only model. To quantify the explanatory power of site, we calculated the likelihood ratio (McFadden's pseudo- R^2):

$$R_L^2 = 1 - \frac{\ln(L_{site})}{\ln(L_{int})}$$

which is the proportional increase in log likelihood L (or decrease in $-2 \log L$) with inclusion of site (L_{site}), relative to an intercept-only model (L_{int}) (McFadden 1974). R_L^2 is analogous to R^2 , the ordinary least squares coefficient of determination, with L_{site} and L_{int} analogous to the residual sum of squares and the total sum of squares, respectively (Menard 2000). Values of R_L^2 between 0.2 and 0.4 indicate strong explanatory power (McFadden 1979). We similarly evaluated variation in the frequency of infection in control replicates, which reflects early-life exposure of juvenile snails prior to collection from the field. We applied the same statistical approach to the 2014 assay, evaluating each of the two parasite sources separately. We discuss comparison of susceptibility to the two sources in the final section of the Methods. For all generalized linear models, we found no evidence of overdispersion, indicating that use of the binomial distribution was appropriate.

We examined the geographic distribution of susceptibility using the same spatial analyses described previously: Moran's I and a Mantel test of geographic distance and overall mean susceptibility (mean of exposed replicates across all years, including both sources in 2014), a GEE with latitude and longitude as predictors (subject: site; within-subject: year, first-order autoregressive) of yearly mean susceptibility (mean of exposed

replicates within a year) (linear response variable), and Spearman rank correlations of yearly mean susceptibility with latitude and longitude for each year. Proportions were arcsine transformed.

Is susceptibility positively correlated with infection prevalence?

We then tested the prediction that sites with highly susceptible juveniles in the lab also display higher infection prevalence in the field. First, we tested for correlations of mean susceptibility and infection prevalence (Pearson; overall and yearly means). We then tested if overall mean susceptibility differed significantly from infection prevalence using a Student's t-test (H_0 : mean difference equal to zero). To examine the contribution of variation in exposure to variation in prevalence, we tested for a correlation of overall mean control infection rates with infection prevalence (Pearson). The infection rate in control replicates reflects variation in early-life exposure of juveniles from different site. Because susceptibility may contribute to variation in infection rate in control replicates, we also performed a partial correlation of overall mean infection prevalence and mean infection rate in control replicates, controlling for susceptibility. This test provides an estimate of the residual variation in infection prevalence between sites that can be explained by variation in exposure alone, after accounting for variation in susceptibility. All proportions were arcsine transformed.

Characterizing variation in susceptibility

In 2014, we exposed hosts to two distinct parasite collections from southern (Source 1) and northern (Source 2) sites, ~4 km apart (Fig. 1A: starred sites). Comparison of susceptibility to these sources allows us to characterize the contribution of host and parasite effects to variation in susceptibility. We were particularly interested in the interaction of host site and parasite source: this interaction explains the majority of variation in susceptibility at larger scales (between lakes) (Lively et al. 2004). Here, we test if this interaction also has explanatory power at a fine spatial scale. We might expect it to be irrelevant given that gene flow within the shoreline habitat is high for host and parasite. We fit a generalized linear model (binomial with logit link function) with host site, parasite source, and their interaction as predictors of the number of infected and uninfected females in exposed replicates. We found no evidence of overdispersion. To test the significance of each effect, we performed likelihood ratio tests of models with and without the effect. Though doses for both sources were likely saturating, any main effect of parasite source may be partly attributed to the lower dose used for Source 1. Lastly, we measured the correlation of mean susceptibility to parasite Sources 1 and 2 at a site (Pearson, arcsine transformed values).

Data are deposited in the Dryad Digital Repository:

<http://dx.doi.org/10.5061/dryad.t89hc> (Gibson et al. 2016a).

Results

Infection prevalence varies in space

Each year, from 2006 to 2015, we measured infection prevalence of *Potamopyrgus antipodarum* with the sterilizing trematode *Microphallus* at 12-13 shoreline sites around Lake Alexandrina, New Zealand. This long-term sampling revealed significant variation in a female's probability of infection between sites (minimum estimated marginal mean = 0.030 ± 0.006 ; 0.030 ± 0.007 SEM at Southwest End and West Bay, respectively; maximum = 0.280 ± 0.020 at JMS) (Fig. 1A) and between years (minimum = 0.080 ± 0.008 in 2014; maximum = 0.20 ± 0.014 in 2009) (Fig. 1B-E). The interaction of site and year was also highly significant, indicating that changes in infection probability between years occur independently at different sites (Table 1, Fig. 1B-E). A female's probability of infection increased significantly with shell length (coefficient = 1.397 ± 0.050 SEM), consistent with an increase in the cumulative risk of infection with snail age (Table 1). There was no significant correlation between geographic distance and mean infection prevalence between sites (Mantel: $r = 0.141$, $p = 0.183$), indicating that sites are independent of one another in space with respect to infection prevalence. In addition, we found no evidence for spatial autocorrelation of mean prevalence (Moran's $I = 0.066$, $p = 0.118$). Relatedly, infection prevalence did not vary significantly with a site's location (GEE: latitude - Wald $\chi^2 = 0.162$, $df = 1$, $p = 0.688$; longitude - Wald $\chi^2 = 2.895$, $df = 1$, $p = 0.089$), indicating no spatial gradient to infection prevalence. Infection prevalence in recent years (mean from 2013-2015) was also uncorrelated with latitude (Spearman's $\rho = 0.363$, $p = 0.223$) and longitude (Spearman's $\rho = 0.286$, $p = 0.344$).

Table 1: Results of generalized linear model for field prevalence of infection.

	<i>Wald χ^2</i>	<i>df</i>	<i>p-value</i>
<i>intercept</i>	1217.7	1	<0.001
<i>site</i>	338.1	12	<0.001
<i>year</i>	97.3	9	<0.001
<i>site*year</i>	270.2	100	<0.001
<i>length</i>	787.4	1	<0.001

Site, year, and their interaction are predictors of the probability that an individual female snail is infected with *Microphallus*. Snails were sampled at 12-13 shoreline sites from 2006-2015. Shell length is a covariate.

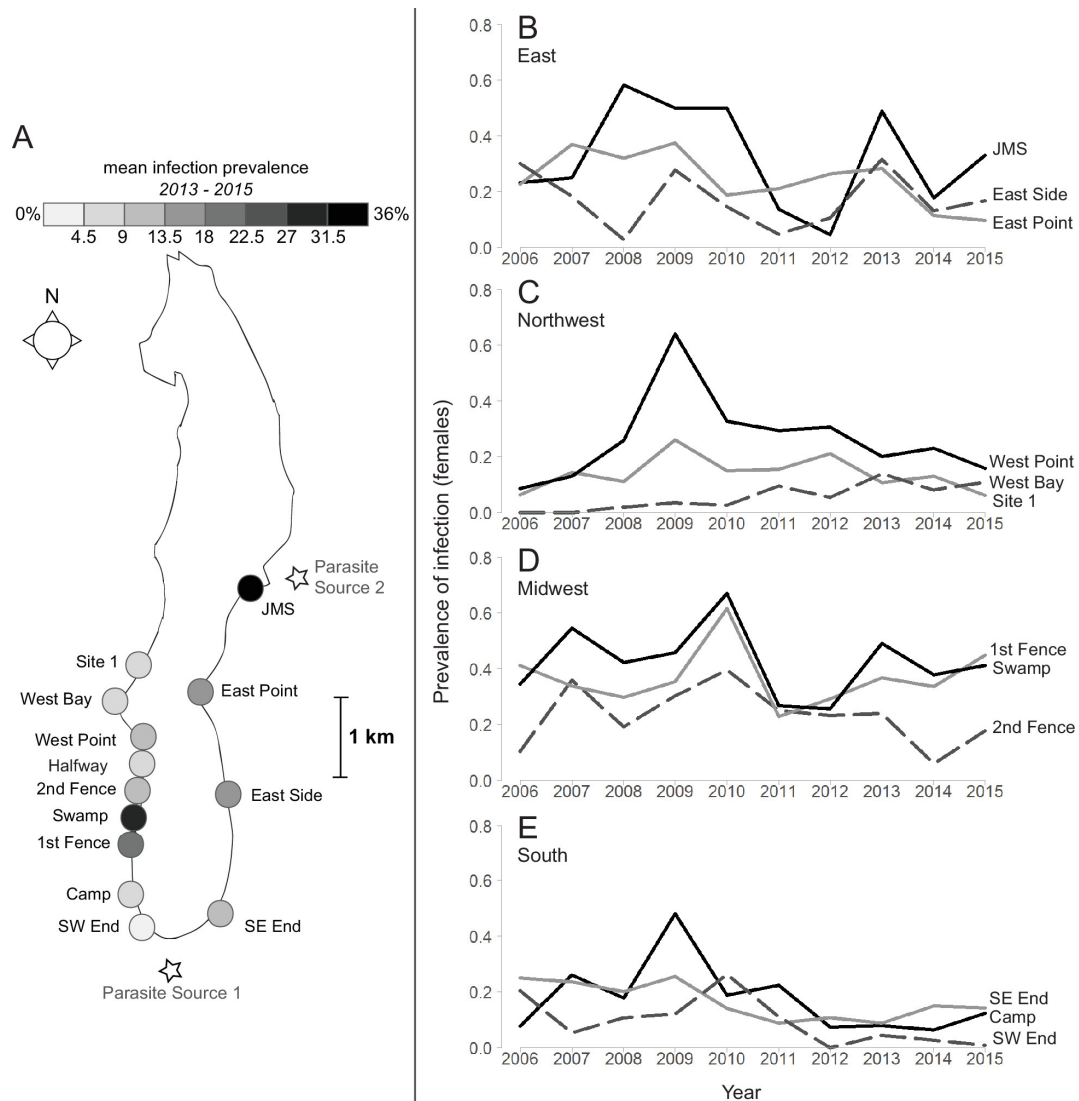


Figure 1: Prevalence of *Microphallus* varies in space and time on a fine spatial scale. (A) Variation in space. Mean prevalence of *Microphallus* in female snails from 2013 to 2015 at 13 sites around Lake Alexandrina (2014-2015 for Halfway). Values are length-corrected estimates derived from a generalized linear model. Stars mark sites where parasites were collected for artificial inoculations in 2014. (B-E) Variation in time. Infection prevalence of adult female snails (> 3.2 mm long) from 2006 to 2015 at 12 sites. Length cut-off was selected to standardize snail age between sites and years. Site Halfway is excluded due to inconsistent sampling.

Susceptibility varies in space

For susceptibility to explain the observed spatial variation in infection prevalence, susceptibility to *Microphallus* must also vary around Lake Alexandrina. We evaluated this prediction through artificial inoculations of juvenile snails collected from sites around the lake. In 2013 and 2015, snails were exposed to a bulk field collection of local parasite eggs. Exposure to field-collected parasites increased the odds of infection with *Microphallus* by 2.2-fold in 2013 (95% CI [1.235, 4.190]; GLM, $z = 2.565$, $p = 0.010$) and by 3.5-fold in 2015 (95% CI [1.504, 9.336]; $z = 2.742$, $p = 0.006$) relative to control replicates (Fig. 2). Given that artificial inoculations were successful, we restricted our analyses to exposed replicates to evaluate variation between sites in susceptibility, measured as the infection frequency obtained in exposed replicates. We found that site contributed substantially to explaining variation in susceptibility, which ranged from 0.210 ± 0.009 SEM at SW End to 0.654 ± 0.038 at East Point in 2013 (GLM, likelihood ratio = 322.940, $df = 11$, $p < 0.001$, $R^2_L = 0.565$) and from 0.150 ± 0.016 at SW End to 0.617 ± 0.007 at JMS in 2015 (likelihood ratio = 123.2, $df = 12$, $p < 0.001$, $R^2_L = 0.361$) (Fig. 2).

In 2014, snails were exposed separately to parasites collected from two different lake sites (Fig. 1A). Exposure increased the odds of infection with *Microphallus* by 1.4-fold for Source 1 (95% CI [1.250, 1.640]; GLM, $z = 5.154$, $p < 0.001$) and by 5.7-fold for Source 2 (95% CI [4.421, 7.499]; $z = 12.959$, $p < 0.001$), relative to control replicates (Fig. 3). Restricting our analyses to exposed replicates, we also found that site contributed substantially to explaining variation in susceptibility (Source 1 – GLM, likelihood ratio = 24.459, $df = 12$, $p = 0.018$, $R^2_L = 0.565$; Source 2- likelihood ratio = 118.31, $df = 12$,

$p < 0.001$, $R^2_L = 0.388$) (Fig. 3). In the final section of the results, we compare susceptibility to these two sources.

Site did not explain variation in infection rate of control replicates in 2014 (GLM: likelihood ratio = 11.063, $df = 12$, $p = 0.524$, $R^2_L = 0.121$) and did so marginally in 2013 (likelihood ratio = 19.239, $df = 11$, $p = 0.0570$, $R^2_L = 0.172$). Site had significant explanatory power in 2015 (likelihood ratio = 30.617, $df = 12$, $p = 0.002$, $R^2_L = 0.260$), suggesting that juvenile snails from different sites did differ in exposure to parasites prior to collection from the field.

We further characterized variation in susceptibility between sites using spatial analyses. There was a significant correlation of overall mean susceptibility with geographic distance between sites (Mantel: $r = 0.318$, $p = 0.029$), suggesting that sites are not spatially independent with respect to susceptibility. We also detected a significant signal of positive spatial autocorrelation for overall mean susceptibility (Moran's $I = 0.234$, $p = 0.001$). Consistent with these findings, mean susceptibility varied significantly with latitude (GEE: Wald $\chi^2 = 6.349$, $df = 1$, $p = 0.012$) and longitude (Wald $\chi^2 = 11.305$, $df = 1$, $p = 0.001$), indicating a spatial gradient in which susceptibility increased from the southwest to the north (coefficient for latitude = 6.332 ± 2.513 SEM) and the east (for longitude: 11.480 ± 3.414). For individual years, we found mixed support for this spatial pattern: mean susceptibility was significantly correlated with latitude (Spearman's $\rho = 0.734$, $p = 0.007$) and longitude ($\rho = 0.608$, $p = 0.036$) in 2013, but marginally so in 2015 (latitude: $\rho = 0.533$, $p = 0.061$; longitude: $\rho = 0.500$, $p = 0.082$) (Fig. 2). In 2014, mean susceptibility to parasite Source 2 increased significantly to the north (latitude: $\rho = 0.610$, $p = 0.027$; insignificant increase to east, longitude: $\rho = 0.484$, $p = 0.094$). Mean

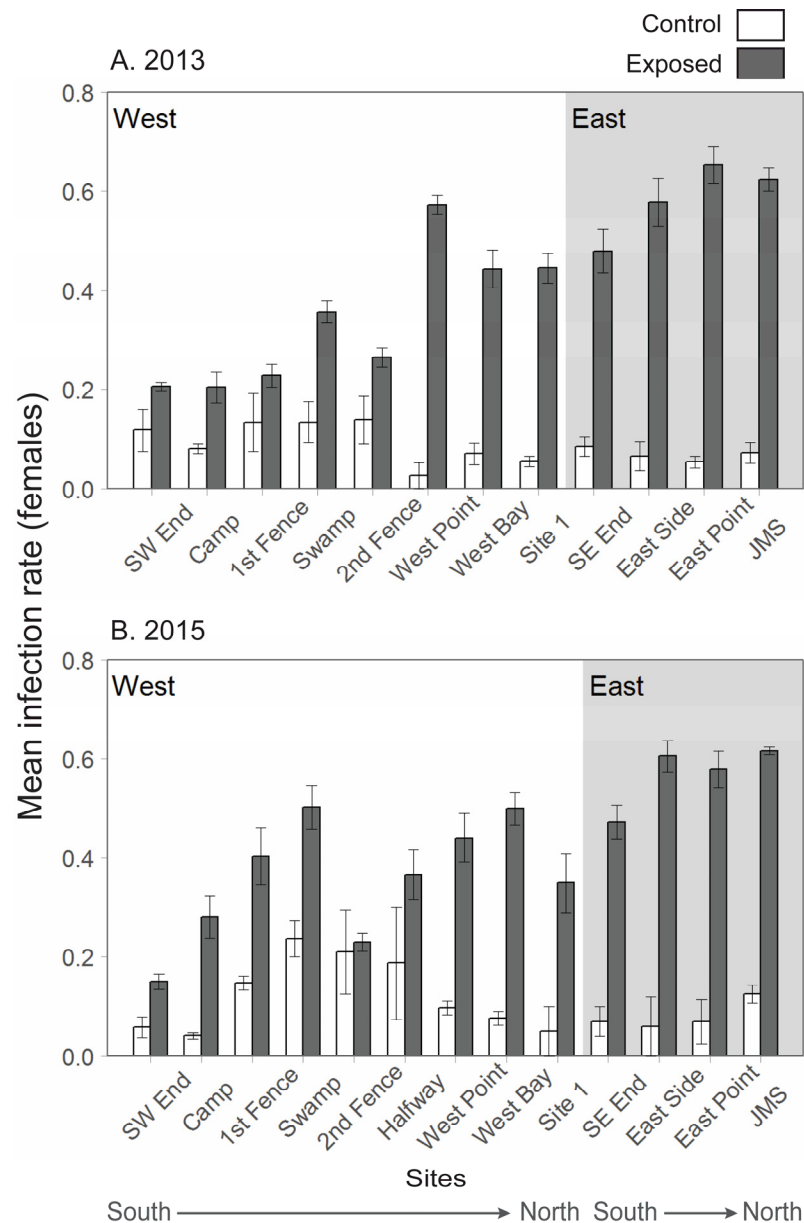


Figure 2: Host susceptibility varies in space. In 2013 (A) and 2015 (B), susceptibility (gray bars: mean infection rate in exposed replicates) differed significantly between the survey sites. Infection rates in the control replicates (white bars) reflect the initial level of infection in field-collected juveniles. These were significantly lower than infection rates obtained in exposed replicates. Susceptibility increased from south to north and from west to east around Lake Alexandrina, particularly in 2013. Sites are grouped by coast (west: left, white background; east: right, gray background) and ordered from southern-most to northern-most within coast.

Results are presented for female snails only. Error bars are standard errors of the means.

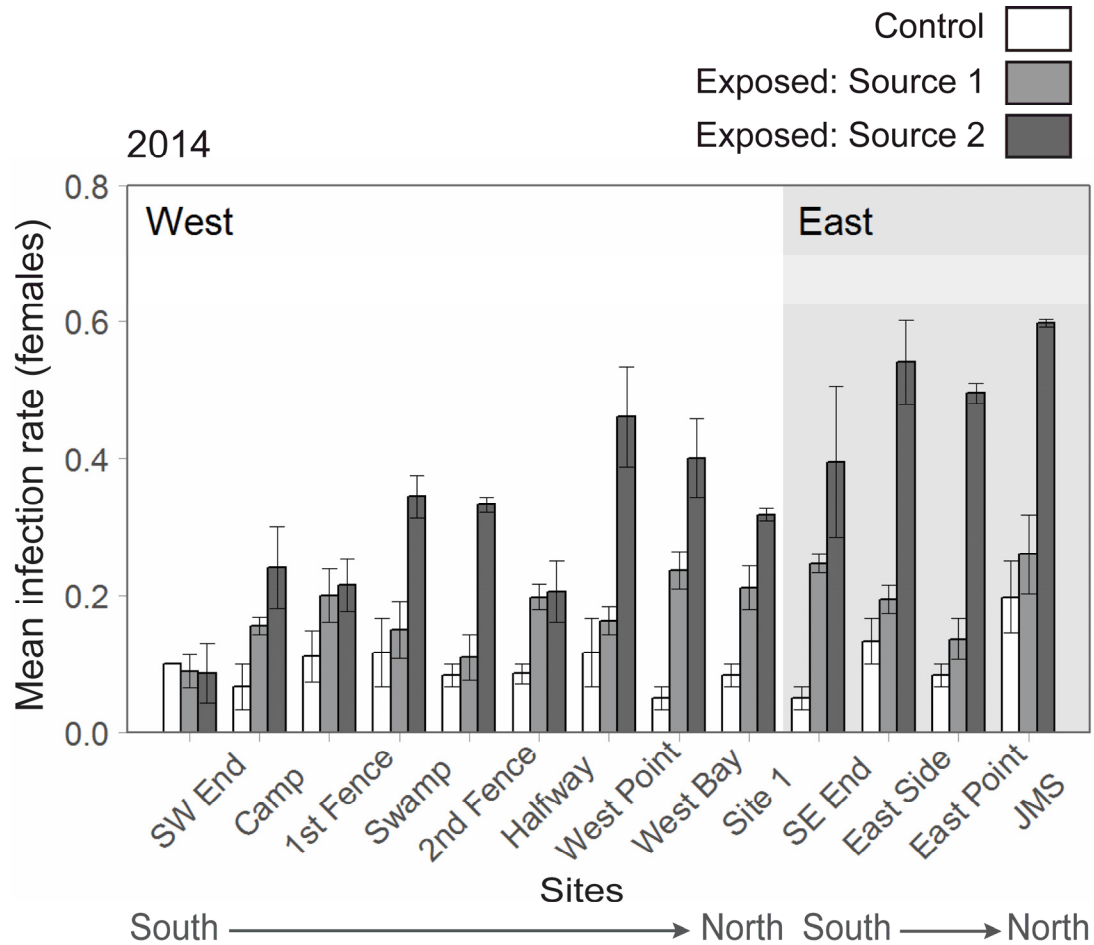


Figure 3: Susceptibility varies by host site, parasite source, and their interaction.

In 2014, artificial inoculations were performed with two distinct parasite sources, Source 1 (southern, light gray) and Source 2 (northern, dark gray). Susceptibility varied significantly with host site, parasite source, and their interaction. Infection rates in the control replicates (white bars) reflect the initial level of infection in field-collected juveniles and were significantly lower than infection rates obtained in exposed replicates of either parasite source. Sites are grouped by coast (west: left, white background; east: right, gray background) and ordered from southern-most to northern-most within coasts. Results are presented for female snails only. Error bars are standard errors of the means.

susceptibility to parasite Source 1, however, showed no spatial gradient (latitude: $\rho = 0.346$, $p = 0.247$; longitude: $\rho = 0.247$, $p = 0.415$) (Fig. 3).

Susceptibility explains observed variation in infection prevalence

If variation in susceptibility explains the observed variation in infection prevalence, we would predict a positive relationship between the two variables. We indeed found a significant positive correlation between overall mean susceptibility to *Microphallus* of hosts and prevalence of *Microphallus* at that site (Pearson's $r = 0.603$, $p = 0.029$) (Fig. 4). Susceptibility can explain 36.4% of the observed variation in infection prevalence between sites. The correlation between mean susceptibility and infection prevalence varied for individual years: it was significantly positive in 2015, marginally so in 2014, and insignificant in 2013 (Table 2). Overall mean susceptibility was significantly higher than mean infection prevalence at a site (one-sample t-test: $t = 9.127$, $df = 12$, $p < 0.001$; mean difference = 0.302 ± 0.033 SEM) (Fig. 4). This is consistent with the high dose of parasites in susceptibility assays relative to natural exposure levels.

Variation in infection prevalence may also arise from variation in exposure between sites, which we estimated as variation in *Microphallus* infection in control replicates (early-life exposure). We did find a significant positive correlation between overall mean rate of *Microphallus* in control replicates and prevalence of *Microphallus* at a site ($r = 0.597$, $p = 0.031$). Mean infection rate in control replicates can explain 35.6% of variation in mean infection prevalence between sites. After accounting for variation in susceptibility between sites, variation in overall mean infection rate in control replicates (field exposure) can explain a large and significant proportion of the residual variation in

infection prevalence (partial correlation: $r = 0.809$, $p = 0.001$, $R^2 = 0.650$). This suggests that, together, variation in exposure and susceptibility (mean infection rates in control and exposed replicates, respectively) can explain the majority of variation in infection prevalence of hosts from different sites.

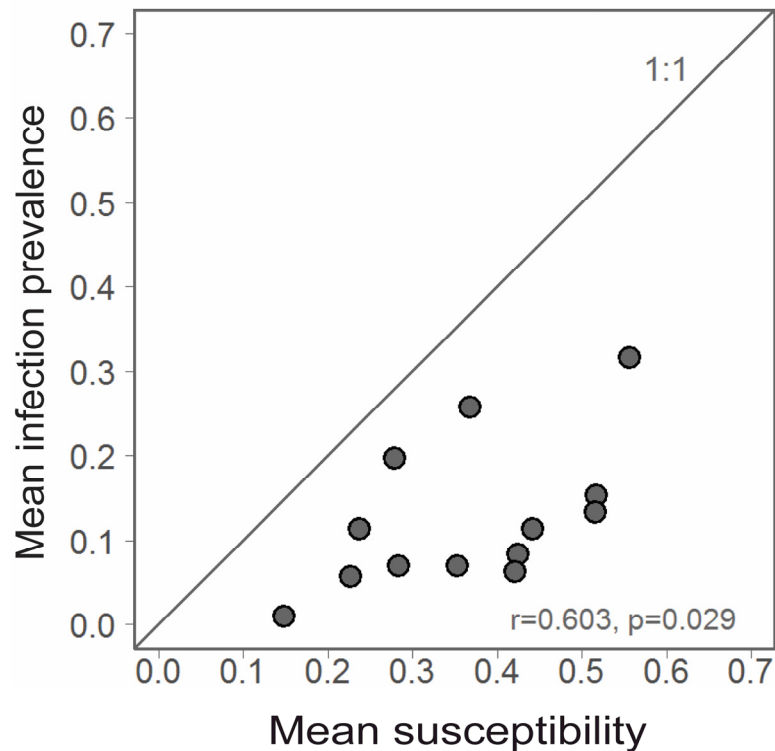


Figure 4: Susceptibility explains spatial variation in infection prevalence. Overall mean susceptibility, measured as mean infection rate in exposed replicates from 2013-2015, is significantly positively correlated with mean infection prevalence. Line indicates a one-to-one relationship of susceptibility and infection prevalence. Susceptibility is consistently greater than infection prevalence in the field. Results are presented for female snails only.

Table 2: Results of correlation of susceptibility and infection prevalence.

	<i>Pearson's r</i>	<i>p-value</i>	<i>R</i> ²
<i>Yearly means: 2013</i>	0.443	0.149	0.196
<i>2014</i>	0.495	0.085	0.245
<i>2015</i>	0.645	0.017	0.416
<i>Overall mean</i>	0.603	0.029	0.364

Results for yearly means in individual years and the overall mean across all years. For 2014, all exposed replicates were included in estimation of susceptibility, regardless of parasite source.

Variation in susceptibility: effects of host, parasite, and their interaction

In 2014, snails from our 13 study sites were separately exposed to parasites collected from southern (Source 1) and northeastern sites (Source 2) of Lake Alexandrina (Fig. 1A: starred sites). By comparing host susceptibility to these two parasite sources, we could determine the proportion of variation in host susceptibility that arises from host main effects (site), parasite main effects (source), and their interaction. We found that both main effects and their interaction made significant contributions to explaining variation in susceptibility (GLM - host site: likelihood ratio = 103.642, df = 12, $p < 0.001$; parasite source: 122.231, df = 1, $p < 0.001$; their interaction: 38.187, df = 12, $p < 0.001$; $R^2_L = 0.435$). The main effect of host site represents variation in susceptibility between host

sites that is independent of parasite source. The main effect of parasite source could reflect inherent variation in infectivity between the two sources, but we cannot rule out the effect of dose, which was lower for Source 1. However, the probability of infection was no greater under exposure to Source 2 vs. Source 1 (odds ratio = 1.2, 95% CI [0.601, 2.25]; $z=0.459$, $p=0.646$). Lastly, the significant interaction effect of host site and parasite source indicates that a proportion of variation in susceptibility arises from the interaction of host and parasite genotypes. Relatedly, host susceptibility at a site to parasite Sources 1 and 2 were not significantly correlated (Pearson's $r = 0.481$, $p = 0.096$).

Discussion

Here, we tested whether spatial variation in infection prevalence is predicted by variation in snail susceptibility to trematode infection, a phenotype with a strong genetic basis (Dybdahl and Krist 2004; Krist et al. 2004; Lively et al. 2004; Koskella et al. 2011). With 10 years of extensive field samples, we demonstrate substantial variation in the prevalence of *P. antipodarum* snails infected with the sterilizing trematode *Microphallus* at shoreline sites distributed along the southern half of a small lake (Table 1, Fig. 1). With experimental inoculations replicated in three years, we demonstrate that susceptibility varies significantly between these shoreline sites (Figs. 2, 3). We then show that variation in susceptibility contributes to variation in fitness in this natural population: variation in susceptibility can explain 36% of variation in mean trematode prevalence between sites (Table 2, Fig. 4). Our findings confirm that infection prevalence can covary with susceptibility at a fine spatial scale.

This covariance is particularly striking given the proximity of our study sites. Rates of migration and gene flow between them are sufficiently high to erode genetic variation at neutral loci (Fox et al. 1996; Paczesniak et al. 2014). Yet the observed variation in susceptibility suggests that loci potentially under selection by parasites are differentiated between these same sites. We identified as much as 6.5-fold variation in the proportion of snails at a site that were resistant to local parasites (2014 – Source 2; Fig. 3). This degree of variation is all the more surprising given that each snail was exposed to very high doses of parasites in our artificial inoculations (as in Osnas and Lively 2004).

The observed variation in susceptibility equals or exceeds that observed between snails from the shoreline and deep-water habitats of Lake Alexandrina (highly susceptible vs. highly resistant, respectively) (Lively and Jokela 1996; King et al. 2009, 2011a). Divergence in susceptibility between these two habitats is perhaps less surprising than the divergence observed here between sites within the shoreline habitat, because genetic divergence between snails from ecologically distinct habitat zones is high relative to that within a single habitat (Fox et al. 1996; Paczesniak et al. 2014). However, the mechanism driving variation in susceptibility is likely the same within and between habitats. Prior work proposes that the depth cline in susceptibility arises from the foraging habits of *Microphallus*'s definitive hosts: dabbling ducks forage in shallow water and so consume shoreline snails, allowing coevolution of shoreline parasites and hosts. They do not forage in deeper water (> 4m deep), preventing coevolutionary cycling and adaptation of local parasites to infect deep water snails (King et al. 2009, 2011a). Variation in duck foraging at shoreline sites may similarly generate a geographic mosaic of coevolution

within the shoreline habitat, such that local parasites differ in the degree to which they are adapted to infect hosts from different sites (as suggested by Vergara et al. 2013).

The geographic covariance of susceptibility and prevalence suggests that parasites can exert strong selection in this system, because genetic variation in susceptibility has clear fitness consequences (i.e. frequency of parasitic castration at a site) (Fig. 4). We emphasize that we are inferring a genetic basis for variation in susceptibility. We could not exclude maternal effects and other variation in condition that might affect host susceptibility, as all experimental snails were collected directly from the field. However, snail susceptibility to trematodes generally has a strong genetic basis (Newton 1953; Richards and Merritt 1972; Richards 1975; Basch 1976; Wakelin 1978; Webster and Woolhouse 1998). In our system, prior studies show that variation in condition does not alter the relative susceptibility of snail genotypes (Dybdahl and Krist 2004; Krist et al. 2004). Additionally, the interaction of host and parasite genotype explains the majority of variation in host susceptibility (43-95% of total variation: Lively et al. 2004), with host populations and clonal genotypes (Lively and Dybdahl 2000; Jokela et al. 2009) typically most susceptible to their sympatric (coevolving) parasites. The most likely explanation for such a pattern is that susceptibility is a product of the specific interaction of host and parasite alleles. Accordingly, hybridization of parasites results in outbreeding depression on sympatric hosts, the best explanation for which is that nonadditive gene effects and genotype specificity underlie susceptibility (Dybdahl et al. 2008). Similarly, experimental selection results in a rapid reduction in susceptibility of hosts that is specific to the experimental parasite population (Koskella et al. 2011). A response to selection is only possible if there is genetic variation for susceptibility. Importantly, there is no evidence

that hosts collected from high prevalence sites are inherently more susceptible to infection: hosts at high and low prevalence sites are equally susceptible to allopatric (non-coevolving) parasites (King et al. 2009, 2011a).

Our results argue that the interaction of host and parasite can explain variation in susceptibility even at the fine spatial scale of our study. Earlier tests of the explanatory power of the host-by-parasite interaction were conducted between lakes (Lively 1989; Lively et al. 2004). The within-lake interaction observed here was unexpected given that rates of gene flow at neutral loci are high for both host and parasite (Dybdahl and Lively 1996; Fox et al. 1996; Paczesniak et al. 2014). Most notably, duck mobility results in little neutral genetic structure for *Microphallus* within and between lakes (Dybdahl and Lively 1996), and yet the within-lake interaction raises the possibility of divergence at infection loci of parasites sampled only four km apart. Both parasite sources are located where ducks breed (AKG and CML, pers. obs.), so localized duck movement during breeding season may promote differentiation of their parasites. Prugnolle et al. (2005) suggest that parasite dispersal may be reduced if migrants are maladapted to infect local hosts. Although local adaptation is evident between lakes, our within-lake interaction was not consistent with local adaptation: north-eastern sites were most susceptible to the north-eastern Source 2 (most susceptible site = JMS: 0.598 ± 0.006 SEM), but southern sites were relatively resistant to the southern Source 1 (least susceptible site = SW End: 0.090 ± 0.024) (Fig. 3). Similarly, host and parasite main effects explained relatively more variation in susceptibility than is typically seen at larger spatial scales (Lively et al. 2004).

We have provided evidence that hosts and parasites diverge geographically, manifesting as spatial variation in susceptibility. The critical question is then: does this variation in susceptibility actually matter for the distribution of infection in nature? Evidence has accumulated for a large role for environmental factors in determining variation in infection prevalence. It is reasonable to assume that environmental variation would overwhelm any contribution of susceptibility to variation in infection prevalence, particularly at fine spatial scales. This is not the case in our system: we find that mean susceptibility can explain over one-third of the variation in mean infection prevalence between sites (Fig. 4). The observed relationship is correlative, but it suggests a link between genetically based host and parasite traits and the distribution of a virulent parasite in a natural population.

Variation in susceptibility, of course, is not the sole explanation for the observed variation in infection prevalence (McNew 1960; Stevens 1960; Scholthof 2007). This is clear from the variable nature of the relationship between susceptibility and infection prevalence: in any given year, the relationship is not consistently significant (Table 2), which may indicate variation in the influence of environmental factors. Most notably, snails at different sites are likely to vary in exposure to parasites, as wind, water movement, and duck behavior may patchily distribute parasite eggs. We find preliminary support for this idea in our examination of control replicates: sites vary somewhat in infection rates of control juveniles, indicative of variation in their exposure to parasites prior to collection from the field. This variation in exposure can explain most of the residual variation in infection prevalence after accounting for susceptibility. We therefore

conclude that susceptibility and exposure together can explain the majority of fine-scale spatial variation in infection prevalence.

Acknowledgements

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Supporting Information

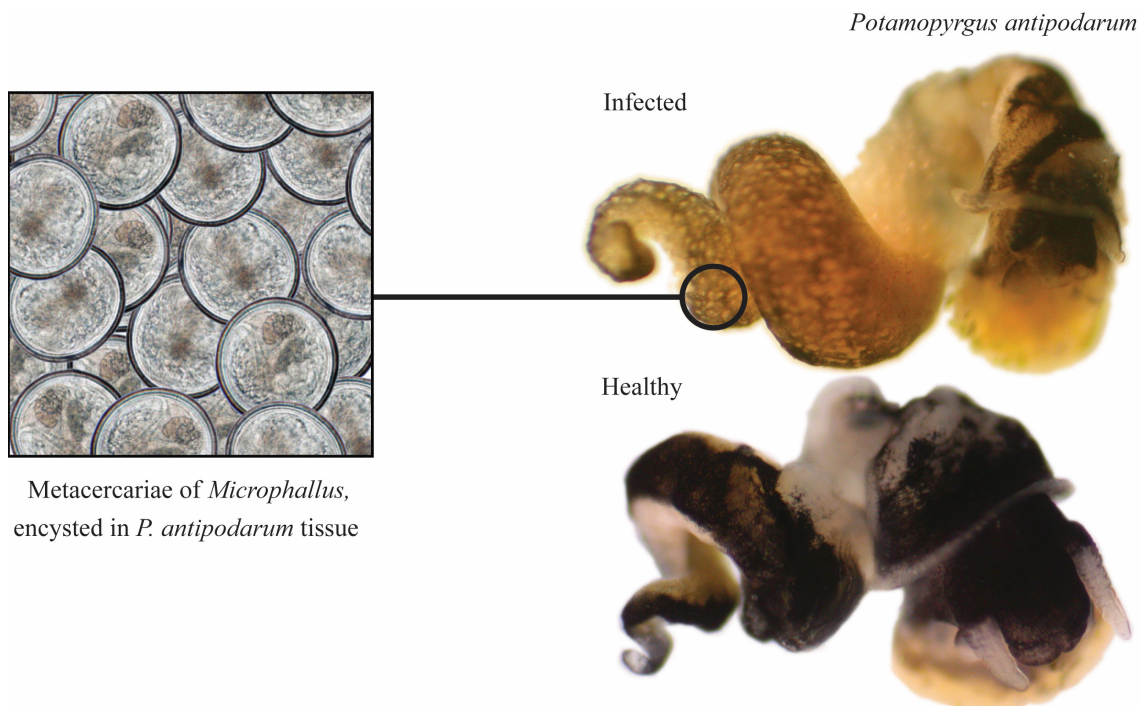
Mortality in susceptibility assays

Data are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.t89hc> (Gibson et al. 2016a).

To determine if high doses of parasites caused snail mortality, we counted the number of snails that survived to the time of dissection (~80 days post-exposure) in exposed and control replicates. Replicates were initiated with 100 snails in 2013 and 75 in 2014 and 2015. Any snails missing at the time of dissection were presumed dead. For each year, we fit generalized linear models to mortality data using the function `glm` in R with the number of dead and surviving snails as a binomial response variable (logit link function) and site, treatment and their interaction as factors. The ratios of residual deviance to residual degrees of freedom exceeded 1, indicating substantial overdispersion (ratio = 3.040 in 2013, 1.886 in 2014, and 4.749 in 2015). Therefore, we re-fit the models using the quasi-binomial distribution, which estimates a scale parameter to account for additional variance in the data (Crawley 2013). Based upon these quasi-binomial models, we used odds ratios and their confidence intervals (function `confint`) to determine if exposure increased the probability of mortality.

We found no evidence that exposure to parasites increased the probability of mortality. In 2013, exposure to field-collected parasites had no effect on the odds of mortality (odds ratio = 0.614, 95% CI [0.303, 1.264]; $z = -0.487$, $p = 0.186$). In 2014, exposure to the relatively low dose of Source 1 decreased the odds of mortality (0.415, 95% CI [0.224, 0.761]; $z = -0.879$, $p = 0.006$), and exposure to the higher dose of Source

2 had no effect (0.663, 95% CI [0.370, 1.187]; $z = -0.441$, $p = 0.171$). In 2015, exposure to parasites had no effect on the odds of mortality (1.069, 95% CI [0.456, 2.504]; $z = 0.067$, $p = 0.878$).



“Stage 5” and “Comparison” by Gabriel Harp, used under Creative Commons BY-SA 4.0 (<http://creativecommons.org/licenses/by-sa/4.0/>). Modified from originals: images combined; line elements and text added.

Chapter 3

Within-population covariation between sexual reproduction
and susceptibility to local parasites

In press as:

Gibson, Xu & Lively (2016) *Evolution*

*“There is, at least at first sight, a serious difficulty with such models.
[They] require the environment to behave in a very odd way...Not only do
environmental features change; correlations between features change. It is
hard to believe that God is as bloody-minded as that.”*

- John Maynard Smith, *Journal of Genetics* (1988), 64: 159-171.

Abstract

Evolutionary biology has yet to reconcile the ubiquity of sex with its costs relative to asexual reproduction. Here, we test the hypothesis that coevolving parasites maintain sex in their hosts. Specifically, we examined the distributions of sexual reproduction and susceptibility to local parasites within a single population of freshwater snails (*Potamopyrgus antipodarum*). Susceptibility to local trematode parasites (*Microphallus* sp.) is a relative measure of the strength of coevolutionary selection in this system. Thus, if coevolving parasites maintain sex, sexual snails should be common where susceptibility is high. We tested this prediction in a mixed population of sexual and asexual snails by measuring the susceptibility of snails from multiple sites in a lake. Consistent with the prediction, the frequency of sexual snails was tightly and positively correlated with susceptibility to local parasites. Strikingly, in just two years, asexual females increased in frequency at sites where susceptibility declined. We also found that the frequency of sexual females covaries more strongly with susceptibility than with the prevalence of *Microphallus* infection in the field. In linking susceptibility to the frequency of sexual hosts, our results directly implicate spatial variation in coevolutionary selection in driving the geographic mosaic of sex.

Introduction

The paradox of sex remains one of evolutionary biology's most fascinating and troublesome questions. Sexual females produce sons, which reduces the per-capita birth rate of sexual lineages relative to asexual ones (two-fold cost of males: Maynard Smith

1971a; Maynard Smith 1978). Given this substantial cost, a mutation to asexual reproduction should rapidly sweep to fixation. Asexuality, however, is rare in eukaryotes (Bell 1982; Suomalainen et al. 1987; Billiard et al. 2012). How can the prevalence of sex be reconciled with its inherent costs relative to asexual reproduction?

Coevolving parasites may confer a selective advantage to sexual females. Known as the Red Queen hypothesis (Hamilton 1975; Levin 1975; Jaenike 1978; Hamilton 1980; Bell 1982), this idea proposes that parasites exert negative frequency-dependent selection by rapidly adapting to infect common host genotypes. Sexual females benefit from the rare advantage, because recombination and segregation can produce offspring with rare genotypes. In contrast, asexual lineages are less able to evade coevolving parasites (Haldane 1949; Jaenike 1978; Hamilton 1980; Hamilton et al. 1990). Thus, if parasites are prevalent and sufficiently virulent, the fitness cost of infection can outweigh the reproductive advantage of asexual lineages, leading to the coexistence of sexual and asexual females, or to the exclusion of asexual females (e.g. Hamilton et al. 1990; Howard and Lively 1994).

The geographic mosaic theory of coevolution provides a powerful framework in which to test the Red Queen in natural systems. One of the core tenets of the geographic mosaic theory is that coevolution is spatially structured, varying in strength across interconnected landscapes (Thompson 1994a; Thompson 1999, 2005). Using this framework, the Red Queen hypothesis makes a strong biogeographic prediction: for species that vary in reproductive mode, the frequency of sexual females should increase with the strength of selection by coevolving parasites (Glesener and Tilman 1978; Jaenike 1978; Lloyd 1980; Bell 1982). Although this prediction seems simple at first

glance, the strength of coevolution is challenging to quantify. Most studies have attempted to quantify coevolutionary selection by measuring infection prevalence, which is simply the proportion of infected hosts (Lively 1987; Johnson 1994; Schrag et al. 1994; Kumpulainen et al. 2004; Ben-Ami and Heller 2005; Meirmans et al. 2006b; Killick et al. 2008; Verhoeven and Biere 2013). Under the Red Queen hypothesis, a positive correlation between infection prevalence and the frequency of sexual females requires that variation in prevalence is correlated with the strength of selection resulting from coevolution (Jaenike 1978). This is likely true at large spatial scales, because variation in prevalence may capture the relatively wide variation in selection among isolated sites (e.g. Levin 1975; Glesener and Tilman 1978; Bell 1982).

Infection prevalence may, however, be too coarse a measure of coevolution at finer spatial scales. From a statistical standpoint, interconnectedness of sites can reduce the variation in selection among sites. Moreover, variation in prevalence can arise from both genetic and environmental factors (McNew 1960; Stevens 1960; Scholthof 2007). In fact, there is substantial evidence that environmental variables influence prevalence, while there is relatively little evidence that variation in prevalence arises from variation in the strength of a coevolutionary interaction (studies reviewed in Little 2002; Gibson et al. 2016b). Environmental “noise” may therefore overwhelm any relationship that may exist between coevolution and prevalence. This is a problem, as one cannot conduct a fair test of the Red Queen hypothesis, or of other coevolutionary hypotheses, without a meaningful measure of coevolution.

We addressed this problem in a natural system that is ideal for studying the evolutionary maintenance of sex. The freshwater snail *Potamopyrgus antipodarum* has

coexisting sexual and asexual forms (Winterbourn 1970), and the frequency of sexual forms is positively correlated with the prevalence of infection by the sterilizing trematode *Microphallus* (Lively 1987; Lively and Jokela 2002; Vergara et al. 2013; McKone et al. 2016). In this system, however, a more precise measure of coevolution is susceptibility, as measured by the proportion of hosts from a given site that become infected following controlled exposure to a fixed dose of *Microphallus*. In inoculations of hosts with parasites from different lakes, most variation in susceptibility (43-95%) arises from the interaction of coevolving host and parasite genotypes (Lively et al. 2004). Snails are more susceptible to their local *Microphallus* population than to foreign ones (Lively 1989; Lively and Dybdahl 2000; Jokela et al. 2009). This pattern of local adaptation arises from strong coevolution, with parasites rapidly adapting to evolutionary change in their local host population (Parker 1985; Lively 1989; Gandon 2002; Lively 2016). Hence susceptibility reflects the strength of coevolution between *P. antipodarum* and *Microphallus*.

If coevolving parasites are responsible for the maintenance of sexual reproduction in *P. antipodarum*, we predicted that 1) sexual snails would be more common at sites where susceptibility to local parasites is high, and 2) the frequency of sexual females would covary more strongly with susceptibility than with infection prevalence. To test these predictions, we measured the covariance between susceptibility and sex for *P. antipodarum*. We found a geographic cline in the frequency of sexual females along the shoreline of a single small lake (6.4 km²), home to an interconnected population of snails (Fox et al. 1996; Paczesniak et al. 2014). Consistent with prediction (1), the geographic distribution of susceptibility to local parasites can explain the majority of variation in sex

within this population, with the frequency of sexual snails increasing with susceptibility. Consistent with prediction (2), susceptibility explains substantially more variation in the frequency of sexual females than does infection prevalence. Our findings specifically support the role of coevolution in the maintenance of sexual reproduction in *P. antipodarum*.

Natural history

The freshwater snail, *Potamopyrgus antipodarum*, is abundant in freshwater streams and lakes of its native New Zealand. Across its native range, a diverse array of asexual clones coexists with sexual males and females, with the frequency of sexual individuals varying from 0 to near 100% (Winterbourn 1970; Lively 1987; Dybdahl and Lively 1995). In lakes with a mixture of sexual and asexual snails, the asexual lineages are commonly derived from the local sexual lineages, consistent with recent and repeated origins of clones (Dybdahl and Lively 1995; Neiman et al. 2005). Reproductive modes are differentiated by ploidy: sexuals are diploid and asexuals primarily triploid (Dybdahl and Lively 1995; higher ploidy levels are occasionally found Neiman et al. 2011). Sexual females and clones are otherwise indistinguishable, having equal fecundities and overlapping ecological niches (Jokela et al. 1997a; Jokela et al. 1997b).

Potamopyrgus antipodarum is the first intermediate host to at least 20 species of digenean trematodes (Hechinger 2012), of which *Microphallus* sp. is particularly well studied and prevalent. The parasite has severe fitness consequences: infected snails are sterilized as their gonads are replaced by larval trematodes (metacercariae) over the

course of ~three months. This is the primary fitness cost of infection, as there is no evidence that *Microphallus* increases the mortality rate of *P. antipodarum* in the field. To our knowledge, the infection process does not vary with snail reproductive mode, as infected male, sexual female, and asexual female snails do not vary inherently in the number and infectivity of metacercariae they produce. Metacercariae are infective to waterfowl, the definitive hosts of *Microphallus*. Ducks ingest infected snails, and *Microphallus* reproduces sexually in their intestines. Within 24-36 hours (pers. obs. CML), infected ducks begin to shed parasite eggs in their feces that are then infective to snails (Hechinger 2012).

This study was conducted at Lake Alexandrina (Mackenzie Basin; South Island, New Zealand). Prior studies have used susceptibility assays to infer variation in coevolution at Lake Alexandrina. Notably, King et al. (2009, 2011a) found that shoreline snails are far more susceptible to Alexandrina's *Microphallus* population than are snails from deep water. They suggested that Alexandrina's *Microphallus* population is able to coevolve with shoreline snails, because ducks forage in shallow water, thereby propagating successful parasite lineages. In contrast, ducks do not dive to deeper water (>4 m), preventing coevolution with deep-water snails. Thus Lake Alexandrina's *Microphallus* population is less able to infect snails from the deep (King et al. 2009, 2011a). Here, we investigate variation in coevolution within the same depth zone along the shoreline of Alexandrina. The shoreline contains an interconnected population of *P. antipodarum*, as evidenced by a lack of structure at neutral loci between distant shoreline sites (Fox et al. 1996; Paczesniak et al. 2014).

Methods

All data generated for this study are archived at the Dryad Digital Repository; DOI: 10.5061/dryad.811h1.

Experimental surveys of susceptibility and the proportion of sexual females

Susceptibility and the proportion of sexual females were obtained from an artificial inoculation experiment replicated over three years. Some of the data from this experiment and the associated methods were initially reported in Gibson et al. (2016b), which was focused on spatio-temporal variation in infection rather than coevolutionary interactions and sex. We summarize the methods here, and provide additional detail on the determination of snail reproductive mode.

In early February of each year, we collected snails from shoreline sites of Lake Alexandrina (12 sites in 2013; 13 in 2014/2015). We isolated juvenile snails (< 2.5 mm in length) for artificial inoculations because they have experienced relatively little exposure in the field (Levri and Lively 1996). We simultaneously collected duck feces, which contain parasite eggs infective to sympatric snails. In 2013 and 2015, duck feces were collected lake-wide and pooled to ensure that the inoculum represented Lake Alexandrina's *Microphallus* population as a whole. In 2014, we were only able to use duck feces from a single site, JMS. Lack of structure at neutral loci suggest that the *Microphallus* population is well mixed (Dybdahl and Lively 1996), but we recently found some evidence for divergence in the parasite subpopulations at different sites (Gibson et

al. 2016b). Use of a single site in 2014 is therefore a conservative measure of host susceptibility.

For each of the 12-13 sites, we divided juvenile snails into experimental replicates of 75 (2014-2015) or 100 snails (2013). In 2013 and 2015, susceptibility and the proportion of sexual females were determined from four experimental replicates per site. In 2014, susceptibility was determined from three replicates, and the proportion of sexual females was determined from six replicates. Snails were exposed to a high dose of parasite eggs to ensure that every host encountered enough parasites to become infected if susceptible (Osnas and Lively 2004). These high doses did not result in excess mortality (Gibson et al. 2016b).

Following parasite development, we dissected each snail to determine gender and infection status. We froze the heads of dissected female snails for flow cytometry analysis, which can distinguish diploid (sexual) from triploid (asexual) females with high confidence (Osnas and Lively 2006) (Supporting Information, SI1). In 2013, we analyzed ~20 female snails per replicate, for a total of 950. In 2014 and 2015, we analyzed up to 30 females per replicate, for totals of 1195 and 1348, respectively. Male snails were not analyzed because they are exclusively diploid at *Alexandrina*'s shoreline (Neiman et al. 2011). Moreover, we restricted our analyses to females, because 1) This allowed comparison of individuals that are identical but for reproductive mode and 2) Males are highly variable in size, behavior, and frequency, making comparisons between sites difficult.

Field-based surveys for infection prevalence

We initially reported infection prevalence data in Gibson et al. (2016), and the full details of field sampling can be found there. Briefly, prevalence was estimated by sampling approximately 100 snails from shallow sites and dissecting them to determine length, gender, and infection status. In 2014 and 2015, we sieved each sample to obtain adult snails (> 2.5 mm) prior to dissection and ran flow cytometry on dissected females to determine reproductive mode. We analyzed ~50 female snails per site, for totals of 664 and 662 in 2014 and 2015.

Statistical analyses

Does the proportion of sexual females vary in space? To evaluate variation in the proportion of sexual females between sites, we used the function `glm` in R v3.2.1 (R Core Team 2013) to fit a logistic model with site, year (factor), and their interaction as predictors of the number of sexual and asexual females in a replicate (binomial response variable, logit link function). To test the significance of an effect, we performed likelihood ratio tests of models with and without the effect. We quantified the overall explanatory power of the model using the following likelihood ratio (McFadden's pseudo- R^2):

$$R_L^2 = 1 - \frac{\ln(L_{full})}{\ln(L_{int})}$$

which provides the proportional increase in log likelihood L of the full model (L_{full}) over the intercept-only model (L_{int}) (McFadden 1974). We infer strong explanatory power for

R^2_L values between 0.2 and 0.4 (McFadden 1979). Data were slightly overdispersed (ratio of the squared Pearson residuals to the residual degrees of freedom = 1.360) (Venables and Ripley 2002). Re-fitting the model with a quasi-binomial distribution (Crawley 2013) did not alter the results, so we report results from the original binomial model for ease of interpretation.

To evaluate the distribution of sex in space, we obtained GPS coordinates for each site in Google Earth. We fit a generalized estimating equation (GEE) in SPSS v21 (IBM; Armonk, NY, USA) with latitude and longitude as covariates of the annual mean proportion of sexual females (subject variable: site; within-subject variable: year) (annual mean = mean of experimental replicates in a single year). GEEs are ideal for the analysis of longitudinally clustered data (Liang and Zeger 1986; Zeger and Liang 1986), because the correlation between measurements taken from a single site at multiple time points can be specified using a first-order autoregressive variance-covariance matrix (Wang and Carey 2003; Ziegler and Vens 2010; Vens and Ziegler 2012). We also tested if nearby sites have similar proportions of sexual females by measuring the correlation of straight-line geographic distance and the absolute value of the difference in overall mean proportion of sexual females (equivalent of Euclidean and Manhattan distances for our data) with a Mantel test in the vegan package v2.3-0 (Dixon and Palmer 2003) in R (Pearson correlation, 999 permutations) (overall mean = mean of experimental replicates in all years). Proportions were arcsine transformed for spatial analyses. To verify the results obtained for experimental juveniles, we evaluated variation in the proportion of sexual females in field collections of adults. We found similar patterns for juveniles and adults (SI2).

Does variation in susceptibility explain variation in sex? If coevolving parasites are responsible for the maintenance of sex, we predicted that sexual females should be more common at sites where susceptibility to parasites is high. Susceptibility varies dramatically between sites (Gibson et al. 2016b) and is based strongly in genetics (Dybdahl and Krist 2004; Krist et al. 2004; Dybdahl et al. 2008; Koskella et al. 2011), particularly in the interaction of host and parasite genotype (Lively et al. 2004; Dybdahl et al. 2008).

We calculated susceptibility in two ways. First, we calculated overall susceptibility: the proportion of females infected in an experimental replicate (as reported in Gibson et al. 2016b). This is an accurate measure of the susceptibility of hosts at a site, because the value from each experimental replicate is based upon a relatively large sample of snails ($43.5 \pm \text{SD } 15.3$). However, susceptibility of all females at a site is a function of the proportion of sexual and asexual females at that site. Any relationship between susceptibility and the proportion of sexual females could thus be confounded by non-independence. We therefore calculated susceptibility in a second way: the proportion of sexual females infected in a replicate. While statistically more sound, the value of sexual susceptibility obtained from each experimental replicate is based upon a smaller sample of snails ($18.5 \pm \text{SD } 6.5$). Accordingly, when calculating mean susceptibility of sexual females at a site, we weighted each replicate by its total number of sexual females. Spatial variation in susceptibility of all females is reported in Gibson et al. (2016b), and spatial variation in susceptibility of sexual females is reported in this article's Supporting Information (SI3).

We conducted all analyses using both calculations of susceptibility, with proportions arcsine transformed. First, we performed a GEE with annual mean susceptibility as a predictor of annual mean proportion of sexual females (subject variable: site; within-subject variable: year). We also conducted Pearson and Spearman rank correlations of overall and annual means of susceptibility and the proportion of sexual females. Finally, we tested for a positive correlation of temporal changes in susceptibility and the proportion of sexual females (Pearson and Spearman). We calculated temporal change by subtracting annual means in 2013 from those in 2015.

Does spatial autocorrelation explain the covariation of susceptibility and sex? Any observed correlation between susceptibility and the proportion of sexual females could arise from correlations between both variables with underlying environmental variables. If so, we predicted that spatial variables would better explain the variation in sex than susceptibility. We used a partial linear regression technique to estimate the amount of variation in sex that can be attributed to susceptibility alone (x), the correlated effect of space and susceptibility (y), and space alone (z) (proportions arcsine transformed). We represented space by longitude, which was a far stronger predictor of spatial variation in the proportion of sexual females than latitude (see first section of Results). Consistent with this result, multiple regressions including susceptibility, longitude, and latitude as predictors were over-fit and did not improve upon models that excluded latitude (model selection in SI4, Table S3). We performed the following ordinary least squares regressions to obtain adjusted R^2 values (unbiased estimator - Peres-Neto et al. 2006):

$$\begin{aligned} \text{proportion}(\text{sexual females}) & \\ &= \beta_0 + \beta_1 * \text{susceptibility} + \beta_2 * \text{longitude} + \varepsilon \end{aligned} \quad [1]$$

$$\text{proportion}(\text{sexual females}) = \beta_0 + \beta_1 * \text{susceptibility} + \varepsilon \quad [2]$$

$$\text{proportion}(\text{sexual females}) = \beta_0 + \beta_1 * \text{longitude} + \varepsilon \quad [3]$$

For [1], the R^2 includes variation explained by x , y , and z . For [2], the R^2 includes variation explained by x and y , and for [3], the R^2 includes variation explained by y and z . We determined the variation attributable to the correlated effect (y) via subtraction: $R^2 [2] + R^2 [3] - R^2 [1] = (x+y) + (y+z) - [x+y+z]$. We then calculated the variation explained by x and z (Legendre and Legendre 1998: pg. 528-536). To estimate the significance of each fraction, we used redundancy analysis ordination with the function `rda` in the package `vegan` (Dixon and Palmer 2003) followed by permutation testing with the function `anova` in R. The significance of fraction y cannot be estimated. We performed variation partitioning for overall and annual means of susceptibility and the proportion of sexual females.

To validate these results, we performed a partial Mantel test (R, package `vegan`) of the correlation between the absolute value of differences in overall mean proportion of sexual females and in susceptibility between sites, controlling for the straight-line geographic distance between sites. Lastly, we fit a GEE with susceptibility, latitude and

longitude as covariates predicting variation in annual mean proportion of sexual females (subject variable: site; within-subject variable: year; arcsine transformations).

Is susceptibility a better predictor of variation in sex than infection prevalence? The Red Queen hypothesis is founded upon the specific genetic interaction of coevolving host and parasite lineages. In the *P. antipodarum*-*Microphallus* system, variation in susceptibility is tightly linked to the interaction of host and parasite genetics (Lively et al. 2004; Dybdahl et al. 2008). In contrast, variation in infection prevalence arises from both susceptibility and environmental factors, with environmental variation explaining ~2/3 of the variation in mean prevalence at the within-lake scale (Gibson et al. 2016). If coevolving parasites are responsible for the maintenance of sexual reproduction in *P. antipodarum*, we predicted that the proportion of sexual females would covary more strongly with susceptibility than with prevalence.

We first tested if sexual females are more common at sites where prevalence is high. Variation in *Microphallus* prevalence between sites from 2013-2015 is reported in Gibson et al. (2016b) (all females). Prevalence values are the estimated marginal means produced by a generalized linear model with shell length of individual females as a covariate (Gibson et al. 2016b). Shell length is positively correlated with snail age, so its inclusion as a covariate controls for age-dependent variation in cumulative infection risk among sites (Jokela and Lively 1995b). Here, we test the relationship between sex and length-corrected infection prevalence of all females (2013-2015) and sexual females only (2014-2015) (proportions arcsine transformed). We conducted Pearson and Spearman

rank correlations of overall and annual mean prevalence and proportion of sexual females. To analyze the relationship across all years, we fit a GEE with annual mean prevalence as a predictor of annual mean proportion of sexual females (subject variable: site; within-subject variable: year).

To compare susceptibility and infection prevalence as predictors of variation in sex, we added annual mean susceptibility as a covariate in this model. We also compared the variation in the proportion of sexual females explained in linear regressions with susceptibility vs. prevalence as predictors (overall and annual means). Comparisons were made using R^2 values and the ratio of the likelihoods of susceptibility vs. prevalence models, with ratios exceeding 1 indicating a greater likelihood of the susceptibility model (models are equivalent in parameter number). Lastly, we conducted a partial correlation of overall mean proportion of sexual females and prevalence, controlling for overall mean susceptibility. In these analyses, susceptibility of all females was compared against prevalence of all females, and susceptibility of sexual females against prevalence of sexual females.

Results

Proportion of sexual females varies in space

In 2013, 2014 and 2015, we surveyed 12-13 sites around Lake Alexandrina for the proportion of sexual females. The proportion of juvenile females identified as sexual (diploid) varied strongly among sites (Fig. 1A) and somewhat between years (minimum =

0.711 \pm 0.016 in 2013, maximum = 0.792 \pm 0.012 in 2014) (Table 1). The ranking of sites with regards to the proportion of sexual females also changed among years (Table 1: interaction effect) (Fig. S1). We identified a geographic cline in sex (Fig. 1B) with the proportion of sexual females increasing strongly from west to east (GEE, longitude: Wald $\chi^2 = 16.957$, df = 1, p < 0.001; coefficient = 18.633 \pm 4.525 SEM) and, to a lesser degree, from south to north (latitude: Wald $\chi^2 = 6.380$, df = 1, p = 0.012; coefficient = 7.250 \pm 2.870). Similarly, geographic distance between sites was correlated with differences in overall mean proportion of sexual females (Mantel: n = 13, r = 0.396, p = 0.004).

Table 1: The proportion of sexual females varies with site, year, and their interaction. We fit a model with the number of sexual and asexual females in an experimental replicate as the binomial response variable. The results are shown in the form of likelihood ratio (D) tests of models with and without the effect of interest. R^2_L value indicates very strong explanatory power of the full model.

	<i>df</i>	<i>D</i>	<i>p-value</i>
<i>site</i>	12	532.11	<0.001
<i>year</i>	2	37.49	<0.001
<i>site*year</i>	23	125.49	<0.001
<i>residual</i>	138		
$R^2_L = 0.497$			

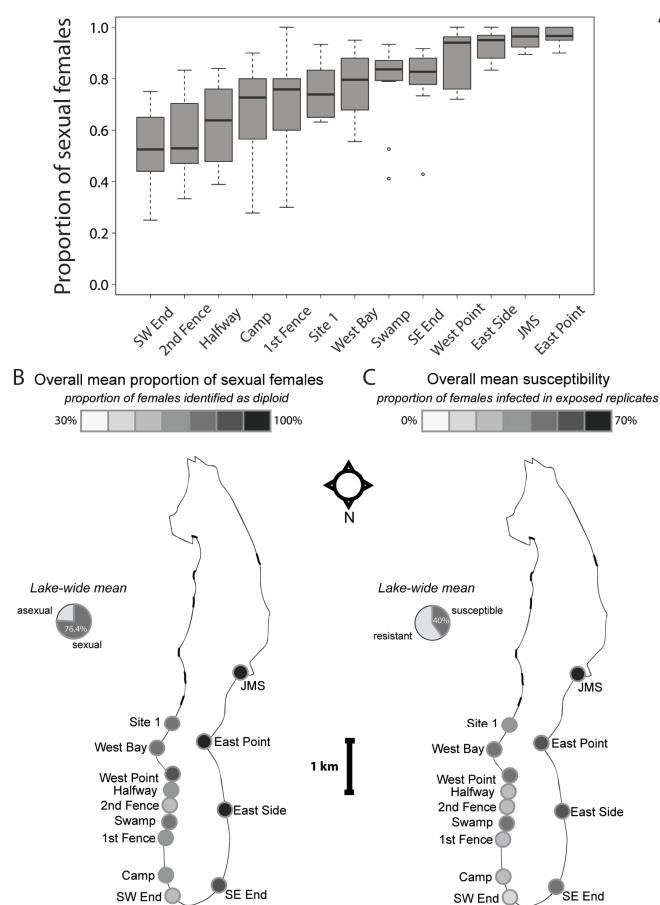


Figure 1: Proportion of sexual females varies in space. (A) The proportion of females identified as sexual (diploids) across all experimental replicates at 13 surveyed sites from 2013-2015. Each site is represented by 14 replicates total: 4 in 2013, 6 in 2014, and 4 in 2015 (exceptions: 13 for East Side and JMS – 3 replicates in 2015; 10 for Halfway – 0 replicates in 2013). Sites are arranged in order of increasing overall mean. Box plots show median (black bar), upper and lower quartiles (limits of box), minimum and maximum (whiskers, excluding outliers), and outliers (dots). (B) Sites around Lake Alexandrina are colored according to the overall mean proportion of sexual females from 2013-2015, demonstrating the north-easterly increase. Pie chart indicates lake-wide mean proportion of sexual females from 2013-2015. (C) The same sites colored according to the overall mean proportion of susceptible females from 2013-2015, demonstrating a similar geographic distribution of susceptibility. Pie chart indicates lake-wide mean susceptibility from 2013-2015. Susceptibility data from Gibson et al. (2016b), where susceptibility was calculated as the proportion of females infected in experimental replicates after exposure to high doses of local parasites.

Variation in susceptibility explains the observed cline in sex

We previously reported variation between Lake Alexandrina sites in susceptibility to local parasites (Gibson et al. 2016b). Susceptibility was measured as the proportion of female juvenile snails that were infected following exposure to high doses of local parasites. As with the proportion of sexual females, we found that susceptibility increased to the north and east of Lake Alexandrina (Fig. 1C).

If coevolving parasites maintain sexual *P. antipodarum*, we predicted that sexual females would be more common at sites where susceptibility was high. Consistent with this prediction, we found that the proportion of sexual females increased with susceptibility at a site (annual means, GEE: all females - Wald $\chi^2 = 144.971$, $df = 1$, $p < 0.001$, coefficient = 1.144 ± 0.095 SEM; sexual females only: Wald $\chi^2 = 42.220$, $df = 1$, $p < 0.001$, coefficient = 0.822 ± 0.127 SEM). Similarly, the mean proportion of sexual females is positively correlated with mean susceptibility in each of the three years (Table 2A,B; Fig. 2A,B). Susceptibility of all females was consistently able to explain the majority of variation in the proportion of sexual females between sites ($0.740 \leq R^2 \leq 0.918$). Susceptibility of sexual females was similarly able to explain a large portion of the variation between sites ($0.342 \leq R^2 \leq 0.654$). These results are supported by more conservative Spearman rank tests (Table S4A,B), except that sexual susceptibility in 2014 was not correlated with the proportion of sexual females under the Spearman rank test (Table S4B).

We next tested if temporal change in the proportion of sexual females at a site is related to change in susceptibility at that site. The change in the proportion of sexual

females from 2013 to 2015 was positively correlated with the change in susceptibility of all females (difference in annual means, Pearson correlation, $r = 0.91$, $df = 10$, $p < 0.001$) and sexual females only ($r = 0.79$, $df = 10$, $p = 0.002$) (Fig. 3). In other words, a decrease in the susceptibility of hosts to local parasites was accompanied by an increase in the proportion of asexual females at that site, while an increase in susceptibility was accompanied by an increase in the proportion of sexual females. These results are supported by Spearman rank tests (all females: $\rho = 0.93$, $df = 10$, $p < 0.001$; sexual only: $\rho = 0.75$, $df = 10$, $p = 0.005$).

Table 2: Results of Pearson correlations of the proportion of sexual females with susceptibility and infection prevalence. We used susceptibility (A,B) and infection prevalence (C,D) for all females (A,C) and sexual females only (B,D) to quantify the strength of coevolutionary selection. Results are shown for annual means in each year and for overall means. Infection prevalence of sexual females was not obtained in 2013. There are 11 degrees of freedom for each correlation ($n=13$), excepting for analyses in 2012 ($df = 10$, $n = 12$).

			2013		2014		2015		Overall mean	
	Predictor		r	p	r	p	r	p	r	p
A	Susceptibility	All ♀	0.93	<0.001	0.86	<0.001	0.96	<0.001	0.96	<0.001
		Sexual ♀	0.77	0.003	0.59	0.036	0.81	0.001	0.77	0.002
C	Prevalence	All ♀	0.38	0.221	0.59	0.035	0.59	0.036	0.57	0.044
		Sexual ♀			0.42	0.152	0.62	0.025	0.47	0.110

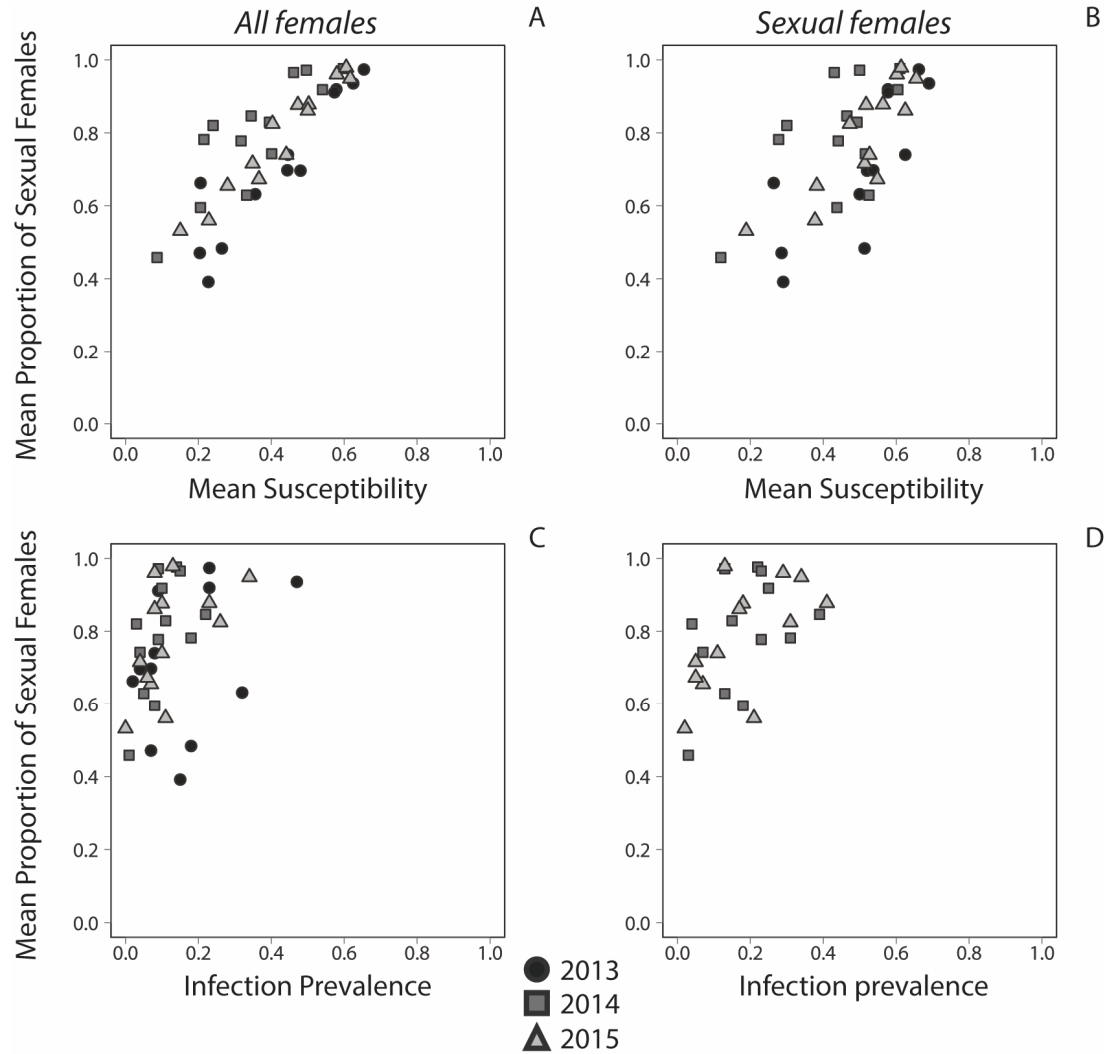


Figure 2: The proportion of sexual females is strongly correlated with susceptibility. We used susceptibility to local *Microphallus* (A,B) and prevalence of infection with *Microphallus* (C,D) to predict variation in the proportion of sexual females. For susceptibility, annual means are shown for all females (A) and sexual females only (B) in 2013, 2014 and 2015, with each point representing one of 13 sites around Lake Alexandrina (12 sites in 2013). The proportion of sexual females was positively correlated with both measures of susceptibility in all years. For prevalence, annual means are shown for all females in 2013-2015 (C) and sexual females in 2014 and 2015 (D). Infection prevalence for sexual females was not measured in 2013. The proportion of sexual females was positively correlated with prevalence of all females in 2014 and 2015 and with prevalence of sexual females in 2015.

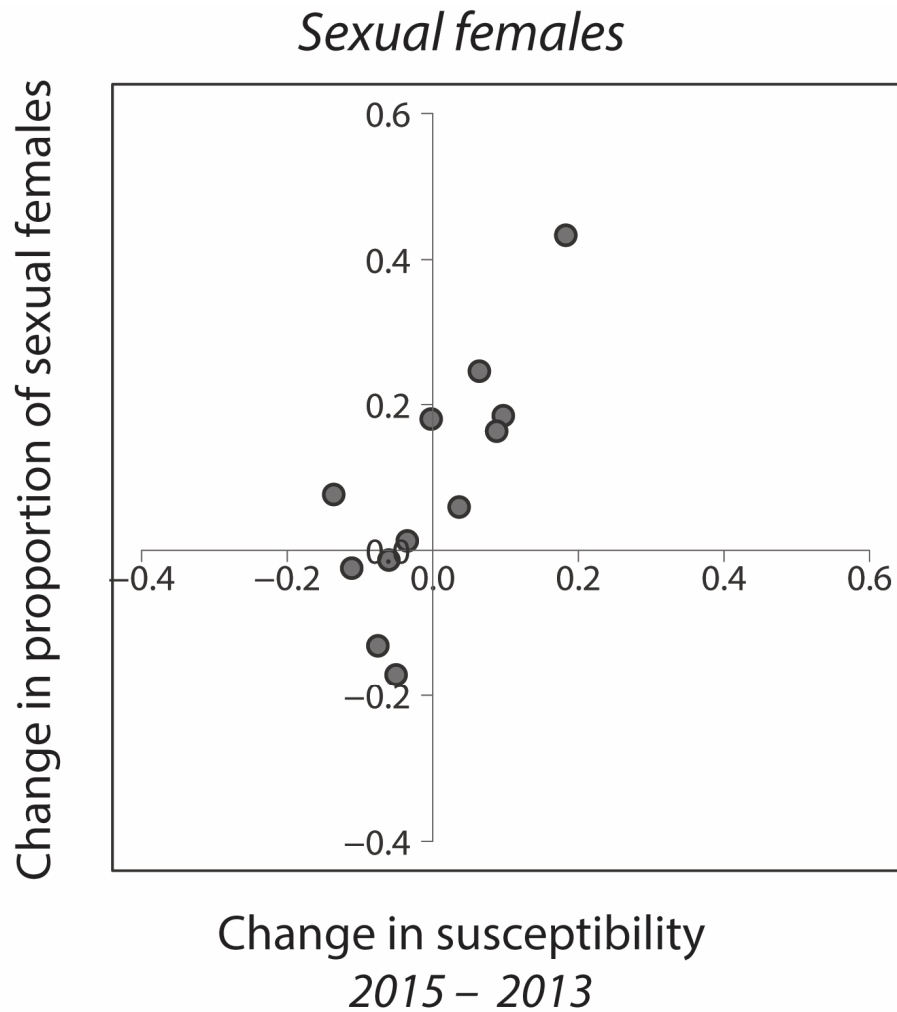


Figure 3: Change in susceptibility to local parasites is positively correlated with change in the proportion of sexual females. Change in susceptibility at a site is shown for sexual females only. Changes are calculated as annual means in 2015 minus annual means in 2013. Each point represents one of 12 sites around Lake Alexandrina (Halfway was not evaluated in 2013).

Correlation of sex and susceptibility does not arise from spatial autocorrelation

Susceptibility and the proportion of sexual females both vary along the same spatial gradient. We therefore used a partial linear regression technique to test the hypothesis that the observed correlation of sex and susceptibility arises from spatial autocorrelation alone. We found no support for this hypothesis. We modeled space using longitude, thus capturing the strong increase in the proportion of sexual females from west to east (Fig. 1b, section 1 of Results). The proportion of sexual females did not vary as strongly from south to north, so we excluded latitude to avoid over-fitting our models (SI4, Table S3). Analyzing all females, the correlated effect of susceptibility and space (y) accounted for the largest portion of explained variation in sex (overall means). Susceptibility alone (x) also accounted for a substantial portion of the explained variation, while space alone (z , represented by longitude) explained a negligible portion (Table 3A). Analyzing only sexual females, we similarly found that the correlated effect y contributed the most to explained variation. Sexual susceptibility alone (x) and space alone (z) each accounted for smaller portions of the explained variation (Table 3B). Analyses of annual means gave similar results: the contribution of space alone to the explained variation in sex was consistently outweighed by the contribution of the correlated effect y plus susceptibility alone (Table S5: components z vs. $x+y$).

Additionally, differences between sites in susceptibility and the proportion of sexual females were strongly correlated when controlling for the geographic distances between sites (overall means, partial Mantel tests, all females: $r=0.858$, $n = 13$, $p=0.001$; sexual only: $r = 0.394$, $n = 13$, $p = 0.009$). Lastly, susceptibility predicted variation in the proportion of sexual females (annual means, GEE, all females: Wald $\chi^2 = 82.725$, $df = 1$,

$p < 0.001$, coefficient = 1.032 ± 0.113 SEM; sexual only: Wald $\chi^2 = 10.251$, $df = 1$, $p = 0.001$, coefficient = 0.461 ± 0.144) when accounting for latitude and longitude. We therefore rejected spatial autocorrelation as the explanation for the strong correlation of susceptibility and the proportion of sexual females.

Table 3: Results of partial linear regressions to account for variation in the proportion of sexual females explained by susceptibility alone (x), the correlated effect of susceptibility and space (y), and space alone (z). Results are given for overall means, with susceptibility calculated using all females (A) and sexual females only (B). Adjusted coefficients of determination (R^2) show the contributions of each component to the total variation explained by the full model. The significance of each component was calculated with redundancy analyses. The significance of y (correlated effect) cannot be calculated. Space is represented by longitude only (SI4).

Average 2013-2015	A. All females		B. Sexual females		
	$adj R^2$	p	$adj R^2$	p	df
<i>Full model (x+y+z)</i>	0.909	0.001	0.705	0.002	2
<i>Unexplained</i>	0.091		0.295		
<i>Uniquely susceptibility (x)</i>	0.358	0.001	0.154	0.026	1
<i>Correlated effect (y)</i>	0.548		0.393		0
<i>Uniquely space (z)</i>	0.004	0.243	0.158	0.026	1

Susceptibility is a better predictor of sex than infection prevalence

The annual mean proportion of sexual females was positively correlated with annual mean infection prevalence of all females in two of three years (Table 2C: 2014, 2015; Fig. 2C) and with infection prevalence of sexual females in one of two years (Table 2D: 2015; Fig. 2D). However, in a full model incorporating all three years, infection prevalence did not predict variation in the proportion of sexual females (annual means, GEE: Wald $\chi^2 = 2.238$, df = 1, p = 0.135, coefficient = 0.210 ± 0.140 SEM), while infection prevalence of sexual females was a marginally significant predictor (Wald $\chi^2 = 3.758$, df = 1, p = 0.053, coefficient = 0.348 ± 0.180).

If coevolution maintains sex, we predicted that the proportion of sexual females would covary more strongly with susceptibility than with infection prevalence. Consistent with this prediction, susceptibility explained a greater proportion of variation in the proportion of sexual females than did infection prevalence (Table 2A,B vs. C,D; Table S6). Accordingly, a linear regression model with overall mean susceptibility as a predictor of the proportion of sexual females was 3.1-fold (all females) and 1.8-fold (sexual females) more likely than a model with mean infection prevalence as a predictor. Analysis of annual means gave similar results (Table S6). After accounting for susceptibility at a site, there was no correlation of overall mean prevalence and the proportion of sexual females (all females: $r = -0.076$, $n = 13$, $p = 0.806$; sexual: $r = 0.084$, $n = 13$, $p = 0.785$), indicating that prevalence can explain no additional variation. For all females, a model including both factors found that susceptibility predicted variation in the proportion of sexual females (annual means, GEE: Wald $\chi^2 = 49.496$, df = 1, $p < 0.001$, coefficient = 0.897 ± 0.128 SEM), but infection prevalence did not (Wald $\chi^2 = 1.401$, df =

1, $p = 0.273$, coefficient = 0.145 ± 0.123). For sexual females only, both factors predicted variation in the proportion of sexual females (annual means, GEE, susceptibility: Wald $\chi^2 = 11.807$, $df = 1$, $p = 0.001$, coefficient = 0.606 ± 0.176 SEM; prevalence: Wald $\chi^2 = 4.754$, $df = 1$, $p = 0.029$, coefficient = 0.338 ± 0.155).

Discussion

Here, we tested the prediction that sexual females are more common at sites where susceptibility to local parasites is high. Consistent with this prediction, three years of replication show that sexual reproduction is tightly and positively coupled with susceptibility to local parasites (Fig. 2, 3; Table 2). A striking geographic cline in the proportion of sexual females at our small study lake (Fig. 1B) is linked to the spatial distribution of susceptibility itself (Fig. 1C), and not to the distribution of any environmental factor correlated with sex and susceptibility (Table 3). We then tested the prediction that the proportion of sexual females covaries more strongly with susceptibility than with infection prevalence. We found consistent support for this prediction. Taken together, our results support the hypothesis that sex is maintained by the specific interaction between coevolving host and parasite genotypes.

For testing coevolutionary hypotheses in the *P. antipodarum*-*Microphallus* system, the distinction between susceptibility and the prevalence of infection is important. Artificial inoculations (Dybdahl and Krist 2004; Krist et al. 2004), hybrid crosses (Dybdahl et al. 2008), and selection experiments (Koskella et al. 2011) all demonstrate that genetic variation in the host and parasite underlies variation in host

susceptibility. Susceptibility also consistently depends upon the combination of host and parasite genotype, at the within (Gibson et al. 2016) and between-lake scale (Lively et al. 2004). In contrast to susceptibility, prevalence of infection in the field has a large environmental component at Lake Alexandrina. In a previous study, we estimated that genetic factors explain approximately one-third of the variation in mean prevalence, while environmental factors explained most of the remaining two-thirds (Gibson et al. 2016b). Susceptibility is therefore a stronger proxy for coevolution than prevalence, particularly at the small spatial scale that we studied here. If coevolving parasites maintain sex, susceptibility should thus covary more strongly with the proportion of sexual females. The opposite result – that prevalence better predicts variation in sex – would undermine the significance of coevolutionary interactions.

We found that the proportion of sexual females does indeed covary more strongly with susceptibility than does infection prevalence. Though there is consistent evidence for a positive correlation between the proportion of sexual *P. antipodarum* and the prevalence of *Microphallus* (Lively 1987; Lively and Jokela 2002; Vergara et al. 2013), we find that correlations with susceptibility tend to be stronger than those with prevalence (Fig. 2, Table 2). Moreover, our correlations with susceptibility are stronger than correlations with prevalence that included ~50% more sites and were thus more powerful (compare to other within-habitat studies: Vergara et al. 2013; McKone et al. 2016). An open question in the field is the extent to which coevolving parasites alone are sufficient to explain the distribution of sex in nature. To tackle that question, our results highlight the importance of selecting a metric that specifically reflects variation in coevolution at the spatial scale of interest.

This problem applies to other host-parasite systems and coevolutionary hypotheses. In general, infection prevalence only coarsely reflects coevolution, because prevalence is commonly linked to environmental factors (e.g. Grosholz 1993; Johnson et al. 2007; Duffy et al. 2012; Altman and Byers 2014; Penczykowski et al. 2014). Even when genetic factors are found to predict prevalence, environmental factors likely also contribute (e.g. Jousimo et al. 2014). Hence, because prevalence is an imprecise measure of coevolution, the lack of a correlation between sex and prevalence cannot automatically falsify the Red Queen hypothesis. Nonetheless, laboratory assays of susceptibility to local parasites may not provide a valid or feasible alternative for quantifying coevolution in all systems.

Our results bring into sharp focus a curious, albeit logical, extension of the Red Queen's prediction. Sexual reproduction enables the production of offspring with rare, resistant genotypes (Haldane 1949; Jaenike 1978). Yet sites with a high proportion of sexual individuals are also those with the lowest proportion of individuals resistant to coevolving parasites (highest susceptibility). While puzzling at first glance, a crucial point is that the Red Queen does not predict that sexual lineages will escape their coevolving parasites altogether. The Red Queen predicts only that sexual lineages will, on average, perform as well or better than asexual lineages when coevolving parasites are present (Hamilton 1980; Vergara et al. 2014). This is quite evident in Figure 3: in a span of only two years, asexual individuals were excluded at sites where susceptibility to coevolving parasites increased. Asexual individuals increased at sites where susceptibility declined, consistent with the idea that common clones can arise in coevolutionary coldspots.

This variation in the strength of coevolution (as inferred from susceptibility) likely arises from the spatial distribution of foraging waterfowl, the definitive host of *Microphallus*. High densities of foraging waterfowl can create coevolutionary hotspots by propagating successful parasite lineages (King et al. 2009, 2011a; Vergara et al. 2013). Various ecological factors may determine the suitability of a site for foraging ducks, such as water depth, wind speed and direction, productivity, and human activity. The patchy distribution of these factors could then explain the observed geographic mosaic of coevolution. Our results argue that this geographic mosaic of coevolution underlies spatial variation in the relative fitness of sexual and asexual reproduction within a single habitat of our small study lake.

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Supporting Information

SII. Flow Cytometry Protocol for Ploidy Analysis

Our flow cytometry protocol is modified from that of Osnas and Lively (2006) and Vergara et al. (2014). Head tissue was individually frozen at -80°C for each dissected female snail. We used exclusively head tissue for ploidy analysis to prevent contamination with parasite DNA. To process for flow cytometry, we ground the frozen sample in 100 μ L of dimethyl sulfoxide (Sigma-Aldrich; St. Louis, MO, USA) and added 275 μ L of propidium iodide solution. This solution consists of 0.006 g propidium iodide (fluorescent dye that binds DNA), 0.014 g spermine (\geq 96%, 0.0208 g spermine tetrahydrochloride, and 30 mL of a detergent solution (3.4 mM trisodium citrate dehydrate, 0.1% Nonidet P-40, 1.5 mM spermine tetrahydrochloride, 0.5 mM Tris) (all reagents from Sigma-Aldrich). We then filtered the sample through 50 μ m mesh (Nitex: CMN-00530-C) into a well of a 96-well cell culture plate. The plate was temporarily stored on ice (15 minutes to 5 hours) to allow debris to settle. We then transferred 150 to

200 μ L of each sample into a new 96-well round bottom cell culture plate, with 50 μ L of PBS per well.

We performed all flow cytometry at the Indiana University-Bloomington Flow Cytometry Core Facility. To measure DNA content, we analyzed 3000 nuclei per sample on an LSRII flow cytometer (BD Biosciences; Franklin Lakes, NJ, USA) with the High Throughput Sampler option, using FACSDiva software v6.1.3 (BD Biosciences). We used a 561-nm laser for excitation of propidium iodide-bound DNA and a 582/15 bandpass filter for detection. We established gates using known sexual and asexual individuals isolated from lab-reared lineages that were originally collected from Lake Alexandrina. These gates were consistent with the ~50% larger genome size of triploid vs. diploid individuals. A small proportion of individuals were excluded from the dataset due to ambiguous DNA content: 4.3%, 4.2%, and 4.4% in 2013, 2014 and 2015 experimental replicates, respectively, and 2.0% and 3.8% in 2014 and 2015 field samples, respectively.

SI2. Proportion of sexual females varies in space: data from field-collected adults

In the Methods section of the main text, we describe the collection of field adults in 2014 and 2015 to estimate infection prevalence. We used the proportion of sexual females in these collections to validate our estimate in the main text of the proportion of sexual females obtained using experimental juveniles. We tested if the proportion of sexual females in field collections varied between sites using a generalized linear model with site and year as predictors of the reproductive mode of individual female snails

(binomial response variable, logit link function) (SPSS v21). There was no evidence of overdispersion, indicating that use of the binomial distribution was appropriate. We tested for a geographic cline in sex using a GEE with latitude and longitude as covariates predicting variation in the proportion of sexual females at a site in each year (subject variable: site; within-subject variable: year – 2014-2015). To test if the proportion of sexual females measured from experimental replicates accurately reflected variation present in the natural population, we compared the proportion of sexual females obtained from experimental replicates to that obtained from field collections using a Pearson correlation of overall mean proportions (2013-2015 for experimental juveniles; 2014-2015 for field-collected adults; arcsine transformation of proportions).

As with experimental juveniles, the proportion of field-collected adult females identified as sexual (diploid) varied between sites (minimum = 0.440 ± 0.050 at Southwest End, maximum = 0.990 ± 0.010 at East Point) (Table S1). A similar geographic cline was present, with the proportion of sexual females increasing to the north (GEE, latitude: Wald $\chi^2 = 6.741$, $df = 1$, $p=0.009$; coefficient = 11.286 ± 4.347 SEM) and marginally to the east (longitude: Wald $\chi^2 = 3.263$, $df = 1$, $p=0.071$; coefficient = 7.493 ± 4.148). These findings resemble those for experimental juveniles. Indeed, the overall mean proportions of sexual females in experimental replicates (2013-2015) and field collections (2014-2015) are tightly correlated (Pearson product-moment correlation = 0.726 , $df = 10$, $p=0.005$), indicating that the observed variation in sex in experimental replicates accurately reflects variation present in the natural population. All analyses in the main text and below rely upon estimates of the proportion of sexual females obtained from experimental juveniles.

Table S1: Results of generalized linear model for variation in the proportion of field-collected adult females identified as sexual. Site and year are predictors of the probability of a female being identified as diploid (sexual) vs. triploid (asexual). Adult females were collected from 12-13 sites around Lake Alexandrina and assayed for ploidy in 2014 and 2015.

	<i>Wald χ^2</i>	<i>df</i>	<i>p-value</i>
<i>intercept</i>	194.324	1	<0.001
<i>site</i>	126.323	12	<0.001
<i>year</i>	2.036	1	0.154

SI3. Sexual susceptibility varies in space

Gibson et al. (2016b) documents spatial variation in overall susceptibility of Lake Alexandrina snails to local parasites. Here, we perform similar analyses for the susceptibility of sexual females. We fit a logistic model with site, year (factor), and their interaction as predictors of the number of infected and uninfected sexual females in experimental replicates (binomial response variable, logit link function) (glm, R v3.2.1). Data were overdispersed (ratio = 1.878). Our results did not change with use of the quasi-binomial distribution, so we report results from the original binomial model for ease of interpretation. To test the significance of an effect, we performed likelihood ratio tests of models with and without the effect. We tested for a geographic cline in sexual

susceptibility (arcsine transformation) using a GEE as described above (covariates: latitude and longitude; subject variable: site; within-subject variable: year – 2013-2015).

Consistent with findings for overall susceptibility, the susceptibility of sexual females varied between sites (minimum = 0.179 ± 0.021 at Southwest End, maximum = 0.570 ± 0.040 at JMS) and years (minimum = 0.334 ± 0.007 in 2014, maximum = 0.504 ± 0.012 in 2013; 0.502 ± 0.010 in 2015), as indicated by their significant contributions to explaining variation in the proportion of sexual females (Table S2). Sexual susceptibility also displayed a geographic cline, increasing to the north (GEE, latitude: Wald $\chi^2 = 9.606$, $df = 1$, $p=0.002$, coefficient = 8.463 ± 2.731 SEM,) and marginally to the east (longitude: Wald $\chi^2 = 3.731$, $df=1$, $p=0.053$, coefficient = 6.526 ± 3.378).

Table S2: The susceptibility of sexual females varies in space and time. We fit a logistic model with the number of infected and uninfected sexual females in an experimental replicate following exposure to high doses of local parasites. Juvenile snails were collected from 12-13 sites and divided into 3-4 experimental replicates for artificial inoculations replicated in 2013, 2014, and 2015. The results are shown in the form of likelihood ratio (D) tests of models with and without the effect of interest. R^2_L value indicates strong explanatory power of the full model.

	<i>df</i>	<i>D</i>	<i>p-value</i>
<i>site</i>	12	123.456	<0.001
<i>year</i>	2	125.540	<0.001
<i>site*year</i>	23	24.422	0.381
<i>residual</i>	138		
$R^2_L = 0.248$			

SI4. Model selection for variation partitioning

We used a partial linear regression technique to partition the explained variation in sex into components attributed to susceptibility alone (x), the correlated effect of space and susceptibility (y), and space alone (z). This approach relies upon a set of multiple linear regressions in which susceptibility and/or space serve as predictors of variation in the proportion of sexual females between sites (annual means for 2013-2015 and overall mean; susceptibility for all females or sexual only).

Space can be represented by two variables, latitude and longitude, resulting in multiple regressions including as many as three predictor variables. Given the small sample size of these analyses ($n=13$ for overall mean and 2014/2015 annual means, $n=12$ for 2012 annual mean), we investigated the potential for overfitting. We consistently found that, for the ordinary least square regressions used in variation partitioning, latitude was an insignificant predictor of variation in the proportion of sexual females when both longitude and susceptibility were included as predictors. Therefore, we compared the global model:

$$\begin{aligned} & \text{proportion}(\text{sexual females}) \\ &= \beta_0 + \beta_1 * \text{susceptibility} + \beta_2 * \text{longitude} + \beta_3 * \text{latitude} + \varepsilon \end{aligned}$$

to the reduced model:

$$\text{proportion}(\text{sexual females}) = \beta_0 + \beta_1 * \text{susceptibility} + \beta_2 * \text{longitude} + \varepsilon$$

without latitude as a predictor.

The adjusted coefficient of determination of the reduced model exceeded that for the full model in all analyses, excepting for 2015 (Table S3). We performed likelihood ratio tests using the function `lrtest` in the package `lmtest` (Zeileis and Hothorn 2002) in R (R Core Team 2013). These consistently indicated that we could not reject the more parsimonious reduced model in favor of the more complex global model (Table S3), though in 2015 the likelihood ratio test found marginal support for the global model. In other words, we could not justify the inclusion of an additional parameter for latitude, because latitude did not explain any additional variation in the proportion of sexual females relative to susceptibility and longitude. This is consistent with our finding that the proportion of sexual females increased far more strongly from west to east than from north to south (Results, first section).

Accordingly, we represented space as longitude, rather than longitude and latitude, in all variation partitioning. Space therefore reflected only the strong west-east cline in the proportion of sexual females. Given the marginal support for the global model in 2015, we performed variation partitioning using both the reduced model and the global model for 2015. The results did not differ, so we report the results for the reduced model.

Table S3: Summary of comparison of global and reduced model. The global model includes latitude as a predictor of the proportion of sexual females. The reduced, parsimonious model includes only longitude and susceptibility (A: all females, B: sexual females only) as predictors.

A. <u>All females</u>		Adjusted R², model:		Likelihood ratio test		
Test		<i>Global</i>	<i>Reduced</i>	<i>X²</i>	<i>df</i>	<i>p</i>
<i>Annual mean</i>	<i>2013</i>	0.8079	0.8291	0.0041	1	0.9487
	<i>2014</i>	0.6555	0.6899	0.001	1	0.9925
	<i>2015</i>	0.9260	0.9155	3.1029	1	0.0782
<i>Overall mean</i>		0.8992	0.9093	0.0004	1	0.9834
B. <u>Sexual females</u>		Adjusted R², model:		Likelihood ratio test		
Test		<i>Global</i>	<i>Reduced</i>	<i>X²</i>	<i>df</i>	<i>p</i>
<i>Annual mean</i>	<i>2013</i>	0.5902	0.6273	0.2746	1	0.6002
	<i>2014</i>	0.3728	0.3802	1.2147	1	0.2704
	<i>2015</i>	0.7719	0.7588	2.097	1	0.1476
<i>Overall mean</i>		0.6884	0.7051	0.6522	1	0.4193

Table S4: Results of Spearman rank correlations of the proportion of sexual females with susceptibility and infection prevalence. We used susceptibility (A,B) and infection prevalence (C,D) for all females (A,C) and sexual females only (B,D) to quantify the strength of coevolutionary selection. Results are shown for annual means in each year and for the overall means. Infection prevalence of sexual females was not obtained in 2013. There are 11 degrees of freedom for each correlation (n=13), excepting for analyses in 2012 (df = 10, n = 12).

			2013		2014		2015		Overall mean	
Prediction			ρ	p	ρ	p	ρ	p	ρ	p
A	<i>Susceptibility</i>	<i>All ♀</i>	0.92	<0.001	0.83	<0.001	0.97	<0.001	0.96	<0.001
		<i>Sexual ♀</i>	0.89	<0.001	0.23	0.448	0.82	0.001	0.86	<0.001
C	<i>Prevalence</i>	<i>All ♀</i>	0.36	0.248	0.63	0.021	0.58	0.037	0.61	0.028
		<i>Sexual ♀</i>			0.41	0.164	0.61	0.026	0.47	0.110

Table S5: Results of partial linear regressions to account for variation in the proportion of sexual females explained by susceptibility alone (x), the correlated effect of susceptibility and space (y), and space alone (z). Results are given for annual mean susceptibility of all females (A,C,E) and sexual females only (B,D,F) in 2013 (A,B), 2014 (C,D), and 2015 (E,F). Adjusted coefficients of determination (R^2) show the contributions of each component to the total variation explained by the full model. The significance of each component was calculated with redundancy analyses. The significance of y (correlated effect) cannot be calculated. Space is represented by longitude only (SI5).

2013	C. All females		D. Sexual females		
	<i>adj R²</i>	<i>p</i>	<i>adj R²</i>	<i>p</i>	<i>df</i>
<i>Full model (x+y+z)</i>	0.829	0.001	0.627	0.011	2
<i>Unexplained</i>	0.171		0.373		
<i>Uniquely susceptibility (x)</i>	0.421	0.001	0.219	0.02	1
<i>Correlated effect (y)</i>	0.422		0.336		
<i>Uniquely space (z)</i>	-0.014	0.668	0.072	0.128	1
2014	E. All females		F. Sexual females		
	<i>adj R²</i>	<i>p</i>	<i>adj R²</i>	<i>p</i>	<i>df</i>
<i>Full model (x+y+z)</i>	0.690	0.001	0.380	0.038	2
<i>Unexplained</i>	0.310		0.620		
<i>Uniquely susceptibility (x)</i>	0.342	0.007	0.032	0.242	1

<i>Correlated effect (y)</i>	0.375		0.250		
<i>Uniquely space (z)</i>	-0.027	0.805	0.098	0.135	1
<hr/>					
2015	G. All females		H. Sexual females		
	<i>adj R²</i>	<i>p</i>	<i>adj R²</i>	<i>p</i>	<i>df</i>
<hr/>					
<i>Full model (x+y+z)</i>	0.915	0.001	0.759	0.002	2
<i>Unexplained</i>	0.085		0.241		
<hr/>					
<i>Uniquely susceptibility (x)</i>	0.441	0.001	0.284	0.001	1
<i>Overlap (y)</i>	0.469		0.339		
<i>Uniquely space (z)</i>	0.006	0.200	0.135	0.021	1
<hr/>					

Table S6: Comparison of susceptibility and infection prevalence. We used susceptibility (A,C) and prevalence (B,D) to quantify the strength of coevolutionary selection using all females (A,B) and sexual females only (C,D). We compared susceptibility and prevalence as predictors of the proportion of sexual females in linear regression models with R^2 values and the ratio for susceptibility vs. prevalence models. Results are shown for annual means in each year and for overall means. Infection prevalence of sexual females was not obtained in 2013. The ratio exceeds 1 in all comparisons, indicating that susceptibility is consistently a better predictor of variation in the proportion of sexual females than is infection prevalence.

			2013		2014		2015		Overall mean	
	Females	Predictor	R^2	ratio	R^2	ratio	R^2	ratio	R^2	ratio
A	All ♀	Susceptibility	0.86	7.91	0.74	2.14	0.92	3.30	0.91	3.13
B		Prevalence	0.15		0.35		0.34		0.32	
C	Sexual ♀	Susceptibility			0.34	1.38	0.66	1.61	0.59	1.77
D		Prevalence			0.18		0.38		0.22	

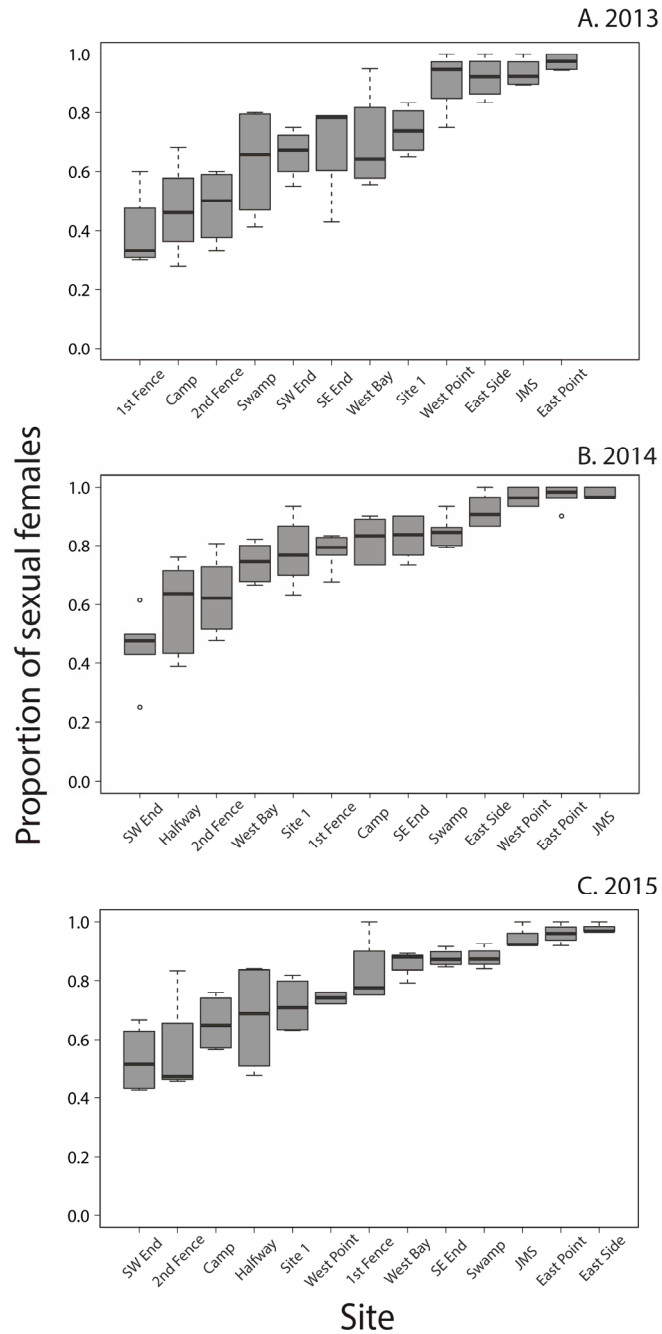


Figure S1: Proportion of sexual females varies between sites and years. The proportion of female juveniles identified as sexual (diploids) in 2013 (A), 2014 (B) and 2015 (C) at 12 (2013) or 13 (2014, 2015) sites. Proportions were obtained from four experimental replicates in 2013 and 2015 and from six in 2014. Sites are arranged in order of increasing mean proportion of sexual females for each year. Box plots show median (black bar), upper and lower quartiles (limits of box), minimum and maximum (whiskers, excluding outliers), and outliers (dots).



Lake Alexandrina (*Taka-moana*), in the Mackenzie Country of New Zealand's South Island. The Southern Alps are in the distance. Taken from Mount John on January 31, 2014 by Amanda Kyle Gibson.

“It is not God, but a parasite, that is being bloody-minded.”

~ John Maynard Smith, *Journal of Genetics* (1985), 64: 159-171.

Chapter 4

A phylogenetic test of the Red Queen Hypothesis: outcrossing
and parasitism in the Nematode phylum

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“Indeed, nothing is more impressive in the biology of parasites than the lengths to which they will go in order to retain amphimixis, or at least cling to some remnant of sexuality.”

~ Graham Bell, *The Masterpiece of Nature* (1982), pages 379-380

Abstract

Sexual outcrossing is costly relative to selfing and asexuality, yet it is ubiquitous in nature, a paradox that has long puzzled evolutionary biologists. The Red Queen Hypothesis argues that outcrossing is maintained by antagonistic interactions between host and parasites. Most tests of this hypothesis focus on the maintenance of outcrossing in hosts. The Red Queen makes an additional prediction that parasitic taxa are more likely to be outcrossing than their free-living relatives. We test this prediction in the diverse Nematode phylum using phylogenetic comparative methods to evaluate trait correlations. In support of the Red Queen, we demonstrate a significant correlation between parasitism and outcrossing in this clade. We find that this correlation is driven by animal parasites, for which outcrossing is significantly enriched relative to both free-living and plant parasitic taxa. Finally, we test hypotheses for the evolutionary history underlying the correlation of outcrossing and animal parasitism. Our results demonstrate that selfing and asexuality are significantly less likely to arise on parasitic lineages than on free-living ones. The findings of this study are consistent with the Red Queen Hypothesis. Moreover, they suggest that the maintenance of genetic variation is an important factor in the persistence of parasitic lineages.

Introduction

Outcrossing, the fusion of gametes from two different individuals, is the most prominent reproductive strategy in Eukaryotes. Uniparental modes of inheritance, including self-fertilization and parthenogenesis, are in contrast quite rare (Bell 1982; Suomalainen et al. 1987; Dacks and Roger 1999; Billiard et al. 2012). Yet outcrossing carries significant

costs. Theory predicts that these costs accrue as either a significantly depressed per-capita growth rate of outcrossing lineages relative to uniparental lineages or as a reduction in relatedness between parent and offspring (Williams 1975; Maynard Smith 1978; Charlesworth 1980; Lively and Lloyd 1990). A paradox thus emerges: how can the prominence of outcrossing be reconciled with its costs?

The Red Queen Hypothesis offers a potential solution. It proposes that antagonistic coevolution between interacting species selects for the maintenance of outcrossing. If coevolving parasites adapt to specifically infect the most common genotypes in a host population, then rare host genotypes gain a fitness advantage by evading parasitism (Haldane 1949; Jaenike 1978). Outcrossing allows for the production of offspring with rare genotypes, while prolonged periods of uniparental reproduction propagate genetically uniform lineages (Hamilton 1980; Hamilton et al. 1990). The Red Queen thus predicts that outcrossing should be maintained in the presence of virulent coevolving parasites. Many empirical studies have supported this prediction by demonstrating that host-parasite coevolution explains the observed distribution of outcrossing in hosts: outcrossing is common in environments or host taxa in which parasite pressure is high (e.g. the tropics, long-lived species) (Bell 1982; Burt and Bell 1987; Lively 1987; Verhoeven and Biere 2013; Wilson and Sherman 2013).

Similarly, the Red Queen predicts that outcrossing should be maintained in the coevolving parasites themselves (Bell 1982). Just as hosts are under selection to evade parasitism through the production of rare genotypes, parasites are under equivalent or greater selection to infect their ever-changing host population (Howard and Lively 2002; Galvani et al. 2003; Salathé et al. 2008; King et al. 2011b). Parasites continually degrade

their environment (the host population) by decreasing the frequency of the common host lineages to which they are adapted. Thus a common parasite genotype with high fitness is predicted to deplete its host lineage and suffer low fitness in later generations. In contrast, a rare parasite genotype has a greater probability of infecting alternate host genotypes and thus gains a fitness advantage. Few empirical and theoretical studies have investigated this prediction (though see Bell 1982; Howard and Lively 2002; Zhan et al. 2007). A related prediction argues that outcrossing is favored in vertebrate parasites because of selection pressure exerted by the rapidly coevolving adaptive immune system. This prediction has received little support thus far (Gemmil et al. 1997; Lythgoe 2000; West et al. 2001) (though see Galvani et al. 2001; 2003).

Bell first formulated the parasite-centric prediction of the Red Queen in his 1982 book *The Masterpiece of Nature*. He argued that under this hypothesis, outcrossing should be more common in parasitic taxa than in their free-living relatives. This prediction was indirectly supported by the difficulty of finding taxa with which to address it: many eukaryotic parasitic groups are invariably outcrossing (e.g. phylum Acanthocephala, subclass Pentastomida) (Bell 1982), impeding a comparative approach. Bell (1982) proposed the Nematode phylum as a uniquely diverse taxon for comparative studies. By comparing the reproductive mode and ecology of different nematode families, Bell (1982) offered tentative support for the Red Queen: he found that outcrossing is common in families that parasitize animals, but less so in plant parasitic and free-living families.

A rigorous evaluation of Bell's (1982) prediction that outcrossing is more common in parasitic taxa than in their free-living relatives requires a phylogenetic

comparison that accounts for the role of shared ancestry in explaining trait distributions. The tools necessary for this phylogenetic approach were not available at the time of publication of *The Masterpiece of Nature*. Since then, molecular and phylogenetic resources have become available for the Nematoda. Using these tools, studies have identified multiple transitions from free-living to parasitism (Blaxter et al. 1998; Dorris et al. 1999; De Ley 2006; van Megen et al. 2009) and from outcrossing to uniparental reproduction in the phylum (Kiontke et al. 2004; Kiontke and Fitch 2005; Cutter et al. 2008; Denver et al. 2011; Kiontke et al. 2011b).

Here, we take advantage of these resources to further test the Red Queen's prediction that outcrossing should be more common in parasitic species than in their free-living relatives. We use a recent phylogeny of the Nematode phylum (Meldal et al. 2007) to make a fine-scale species-level comparison of parasitic and free-living taxa while accounting for shared ancestry. Adding to Bell's preliminary results, we find that the Red Queen Hypothesis successfully explains the macroevolutionary distribution of outcrossing. While outcrossing is maintained in lineages of parasitic nematodes, notably in animal parasitic lineages, free-living lineages are susceptible to invasion by uniparental modes of reproduction.

Methods

Phylogeny

B. Meldal provided the phylogenetic reconstructions from Meldal et al. (2007), which were based upon small subunit ribosomal DNA for 212 taxa distributed across the

phylum. Bayesian inference in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) produced 2700 trees.

For comparative analyses, we removed the closely-related marine clades Desmorida, Chromadorida, and Monhysterida (n=48 taxa). Marine taxa are poorly studied (Meldal et al. 2007), and reproductive mode was difficult to ascertain. An additional two taxa, *Calyptronema maxweberi* and one identified only as a marine Tylenchid, were removed due to lack of character data. Marine nematodes should be the focus of future study: they are thought to be largely outcrossing (Bell 1982).

Pruning in Mesquite v2.75 (Maddison and Maddison 2011) produced a phylogeny of 162 species with *Turbanella cornuta* as an outgroup. Pruned trees were made ultrametric using maximum likelihood optimization with the package phangorn v1.99-5 (Schliep 2011) for R v3.0.2 (R Core Team 2013). For this purpose, we used the original sequence alignments and the TIM2+I+G model, which was selected in jModelTest under the Bayesian information criterion with the BIONJ setting (Guindon and Gascuel 2003; Darriba et al. 2010).

Character assignments

For taxa in their phylogeny, Meldal et al. (2007) reported lifestyle, which is the term used here to distinguish free-living from parasitic taxa. We adopted their lifestyle characterizations, with two exceptions supported by the literature. Reproductive mode was determined through literature reviews, personal communication with experts, and searches of databases (Plant and Insect Parasitic Nematodes (<http://nematode.unl.edu/>), Nemaplex (Ferris 1999), Worm Bazaar (Carter and De Ley 2005), WormBook

(<http://www.wormbook.org>), Worm Systematic Resource Network (Fitch 1998)). For taxa identified by genus alone, character states were assigned based upon the genus's type or best characterized species.

For reproductive mode, taxa were classified as having an outcrossing or uniparental mode. Uniparental encompasses both parthenogenesis (strict asexuality) and androdioecy (hermaphrodites self-fertilize and occasionally outcross with rare males). For many taxa, male frequency was the primary factor in determining reproductive mode, with rare or absent males indicating uniparental reproduction (Maupas 1900; Triantaphyllou and Hirschmann 1964). Because male rarity cannot distinguish parthenogenesis from androdioecy, these two modes were merged under uniparental reproduction (see Table S1 for details). Theory suggests that the benefits of outcrossing may be obtained by even the rare outcrossing observed for androdioecious nematodes (Hurst and Peck 1996; Agrawal and Lively 2001; Barrière and Félix 2005). Thus the combination of androdioecy and parthenogenesis is conservative for evaluating our hypothesis.

Ancestral states were estimated with stochastic character mapping (Nielsen 2002; Huelsenbeck et al. 2003) in SIMMAP v1.5 (Bollback 2006) using the posterior distribution of trees.

Correlated evolution

We used two different phylogenetic comparative methods to test the hypothesis that outcrossing and parasitism are correlated. Each method allows for different analyses, and support for a pattern is greatly strengthened when similar results are obtained using

approaches with distinct theoretical frameworks. First, we used stochastic character mapping to measure the correlation between outcrossing and parasitism, a general classification encompassing both plant and animal parasitism. Because this approach allows multistate characters, we also tested the more specific hypothesis that outcrossing and animal parasitism are correlated. Secondly, we used the Discrete method to determine if transitions in lifestyle and reproductive mode are correlated (Pagel 1994). We restricted this analysis to free-living and animal parasitic taxa, excluding plant parasites. This approach estimates transition rates between character states, so we also compared transition rates to test two hypotheses for the observed evolutionary patterns. Basic deviations in character distributions were investigated using chi-square tests in R.

1. Stochastic character mapping

Stochastic character mapping was implemented in SIMMAP. This method creates stochastic character maps by sampling the posterior distribution of trees and model parameters. Stochastic character maps provide a posterior distribution of character histories with which to quantify character correlation (Nielsen 2002; Huelsenbeck et al. 2003; Bollback 2006). The method measures the observed frequency of co-occurrence of states i and j across character maps and their expected co-occurrence given the frequency of each state. The statistic d is the deviation of observed from expected. Positive values indicate greater co-occurrence than expected, while negative values indicate less than expected. Significance is determined by sampling d from character maps constructed under the assumption that character states are not associated. The probability of the

observed d value is measured against this null distribution (Huelsenbeck et al. 2003; Bollback 2006).

SIMMAP accounts for uncertainty in modeling character evolution by assigning priors on the parameters for each trait (Schultz and Churchill 1999; Bollback 2006). Prior parameters were obtained using SIMMAP's Markov chain Monte Carlo (MCMC) analysis to generate a posterior distribution for the overall rate of character evolution (under a gamma prior) and the bias parameter (under a beta prior for two-state characters or an empirical prior for three-state characters). Posterior distributions were analyzed in R using the SIMMAP script (<http://www.simmap.com/pgs/priors.html>) to obtain the best-fitting parameters. Prior parameters were determined independently for each analysis using the consensus phylogeny.

Analyses of character correlation were performed using the 2700 trees, rooted and with outgroup excluded. 100 samples, prior draws, and predictive samples for significance measures were taken. Specific analyses are outlined in Table S3.

2. Discrete method

The Discrete method (Pagel 1994) was implemented in BayesDiscrete within BayesTraits v1.0 (Pagel and Meade 2006). BayesDiscrete tests if the evolution of two binary traits is best explained by a model of dependent or independent evolution. Under dependent evolution, transitions in lifestyle depend upon the state of reproductive mode, and vice versa. Under independent evolution, transitions in lifestyle are independent of reproductive mode, and vice versa (Pagel 1994; Pagel and Meade 2006). The Bayesian version allows for two comparisons of competing models (dependent vs. independent).

First, we used a Bayes factor of the marginal likelihood of competing models (Kass and Raftery 1995). The marginal likelihood is approximated by the harmonic mean of the likelihoods in a very long Markov chain. Second, we compared the proportion of visits made to independent vs. dependent models under the dependent mode of the reversible-jump MCMC. Of the 21,146 models possible under this analysis, 51 (0.24%) are consistent with independent evolution. Therefore, if 0.24% of visits by the reversible-jump MCMC analysis are to independent models, the odds of dependent vs. independent models of evolution are equivalent (Pagel and Meade 2006).

Plant parasitic lineages were excluded from the dataset so as to test correlated evolution of animal parasitism and outcrossing. The 2700 trees were rooted and outgroup excluded. Each analysis was run for 100,050,000 iterations, with a burn-in of 50,000 iterations, sampling every 300 iterations. A reversible-jump gamma hyperprior was used, with parameters seeded from uniform distributions on the interval 0 to 10. A rate deviation parameter of 9 was chosen to obtain an average acceptance rate of 20-40%. Because the harmonic mean of the likelihood may have very large variance and can thus be unstable, five runs of both the independent and dependent analyses were performed (Newton and Raftery 1994; Pagel and Meade 2006; Raftery et al. 2006).

If dependent models of evolution are supported, the underlying transition rate parameters of the dependent analysis can be examined (Pagel and Meade 2006). We tested specific hypotheses by comparing the posterior distributions of the following transition rates: q_{13} with q_{24} , outcrossing to uniparental reproduction on free-living and animal parasitic backgrounds, respectively; q_{13} with q_{12} , free-living to parasitism on an

outcrossing background; and q_{12} with q_{34} , free-living to parasitism on outcrossing and uniparental backgrounds, respectively.

Reversals from uniparental reproduction to outcrossing may be rare, even impossible (Igic et al. 2006; Goldberg and Igic 2008). An additional analysis was therefore conducted with the transition rate from uniparental reproduction to outcrossing (q_{31} and q_{42} in dependent models; beta1 in independent) restricted to 0. Each analysis was run for 1,000,100,000 iterations, with a burn-in of 50,010,000 iterations, sampling every 600 iterations. A gamma hyperprior was used, with parameters seeded from uniform distributions on the interval 0 to 5. A rate deviation parameter of 5 was chosen. Specific analyses are outlined in Table S4.

Both comparative methods described above rely upon a Markov process that Maddison and FitzJohn (2014) argue is flawed. The crux of the problem lies in the assumption of the Markov process that small branch segments are independent. They particularly cite as problematic datasets in which transitions in a character are rare and/or concentrated in a single lineage. In the supplement, we therefore report the methods and results for estimation of transition numbers. Maddison and FitzJohn (2014) also cite nonrandom sampling of characters as a contributing problem. We address this issue in the supplement by measuring correlations under simulations of different sampling schemes.

Results

Ancestral states for reproductive mode and lifestyle

The phylogeny used in comparative analyses was modified from Meldal et al. (2007) and comprises 162 nematode species (Fig. 1). Character states are summarized in Table 1 and detailed in Table S1. Ancestral states were estimated on the posterior distribution of trees using stochastic character mapping. For lifestyle, a free-living ancestor is strongly supported (probability: 99.5%). For reproductive mode, outcrossing as the ancestral state is weakly supported (probability: 64.2%). We find support for multiple transitions between states for both lifestyle and reproductive mode (Table S2).

Table 1: Character states for lifestyle and reproduction of 162 nematode species

		Lifestyle			Totals
		<i>Free-living</i>	<i>Animal Parasite</i>	<i>Plant Parasite</i>	
Reproduction	<i>Outcrossing</i>	48	45 ^b	23	116
	<i>Uniparental</i>	31	0	15	46^d
	Totals	79^a	45	38^c	

^a Sixteen species, identified as free-living in Meldal et al. (2007), are reported to have facultative associations with vertebrate (n=2) or invertebrate (n=14) hosts, including parasitism, phoresy, and commensalism. Given the uncertainty regarding the nature of these associations, these taxa are treated as free-living unless otherwise noted (Table S1).

^b Two animal parasites, *Heterorhabditis bacteriophora* and *Strongyloides ratti*, have unusual life cycles in which selfing and parthenogenesis, respectively, typically alternate with biparental outcrossing. These two species are treated as outcrossing unless otherwise noted (Table S1).

^c Fourteen taxa reported as plant parasites in Meldal et al. (2007) are unlikely to be obligate plant associates. They are commonly reported as soil-dwelling nematodes, in some cases isolated in the vicinity of plant roots. These taxa are treated as plant parasites, in order to adhere to the reported lifestyle, unless otherwise noted (Table S1).

^d Fourteen of these uniparental species are parthenogenic, and four are androdioecious. The remaining 28 species are broadly classified as uniparental (Table S1).

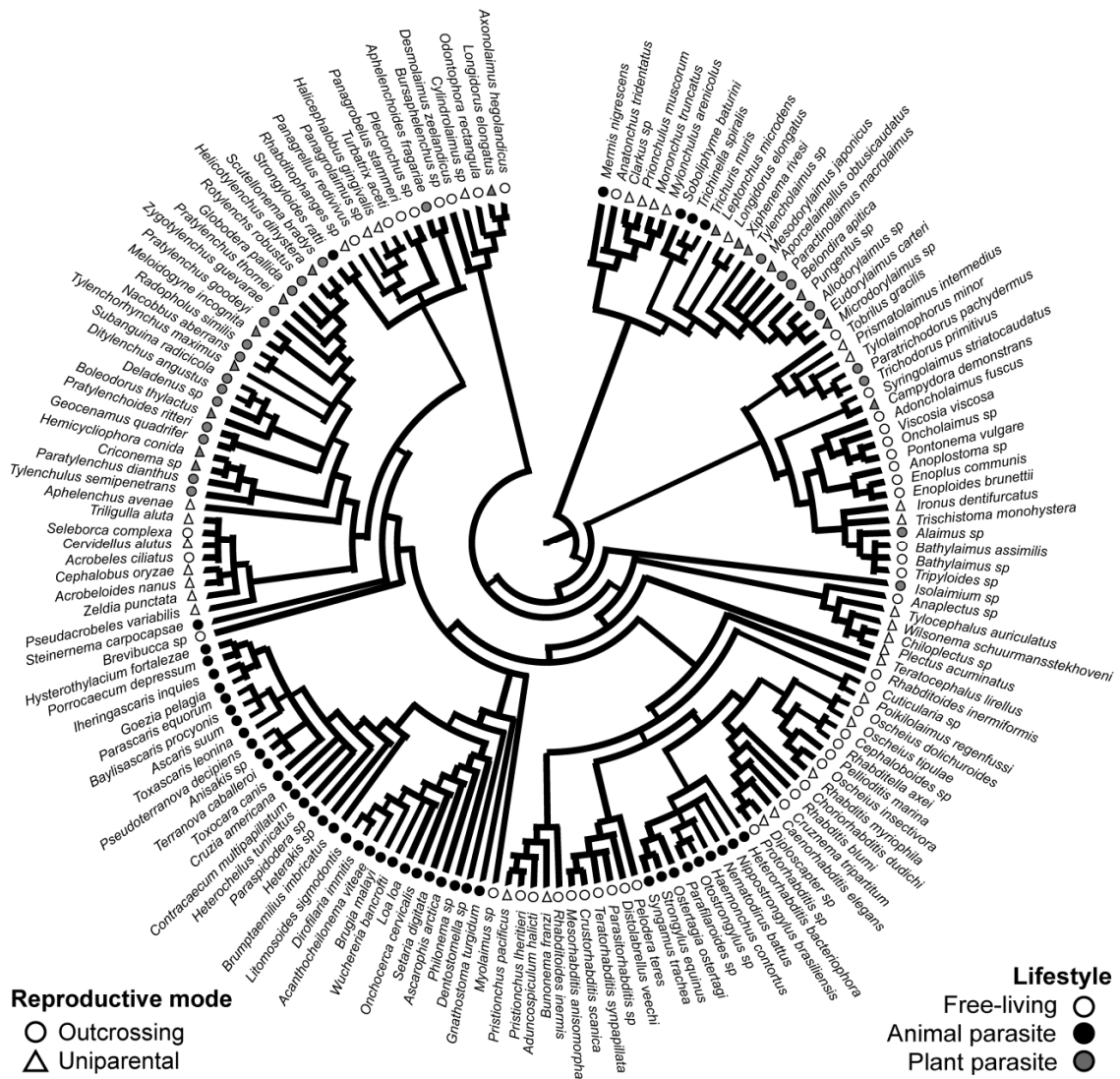


Figure 1: Majority-rule consensus tree and character distribution of 162 species in the Nematoda. This reconstruction represents the consensus of 2700 Bayesian-inferred trees (modified from Meldal et al. 2007). Pruning of the original tree is described in the Methods section. Further modifications were performed in MEGA 5.1 (Tamura et al. 2011). Symbol fill indicates lifestyle: free-living (open), animal parasite (black), and plant parasite (gray). Symbol shape indicates reproductive mode: outcrossing (circle) and uniparental (triangle).

Correlation between outcrossing and parasitism

1. Stochastic character mapping

In our dataset, the proportion of parasitic species that are outcrossing significantly exceeds that predicted by the joint probabilities of parasitism and outcrossing in the dataset ($\chi^2=7.91$, $df=1$, $p=0.005$). This excess of outcrossing holds when accounting for phylogeny: outcrossing and parasitism are significantly positively associated in the evolutionary history of the Nematoda ($d=0.011$, $p=0.02$)(Table S3).

The excess of outcrossing in parasitic species is driven by animal parasites: 100% are obligate outcrossers ($n=43$) or have an outcrossing stage in their life cycle ($n=2$). In contrast, only 60.5% of plant parasites are outcrossing, which is equivalent to the proportion observed in free-living taxa (60.8%)($\chi^2=0$, $df=1$, $p=1$). Indeed, a test for correlated evolution contrasting taxa that are free-living, parasitic on animals, or parasitic on plants finds that outcrossing is significantly negatively correlated with free-living ($d=-0.009$, $p<0.001$), significantly positively correlated with animal parasitism ($d=0.011$, $p<0.001$), and not correlated with plant parasitism ($d=-0.002$, $p=0.19$). The correlation of outcrossing and animal parasitism is unchanged when 16 species that facultatively associate with animal hosts are treated as animal parasites ($d=0.010$, $p<0.001$). The lack of correlation between outcrossing and plant parasitism is similarly unchanged when 14 taxa that are questionably reported as plant parasites are treated as free-living ($d=0.001$, $p=0.31$). The results are also insensitive to assignment of *Strongyloides ratti* and *Heterorhabditis bacteriophora* as outcrossing or uniparental (Table 1, S3).

2. Discrete method

We then used the Discrete method to determine if evolutionary transitions in lifestyle and reproductive mode are correlated. Given the above results, we excluded plant parasites and limited this analysis to free-living and animal parasitic taxa. This approach further supports correlated evolution of outcrossing and animal parasitism. The estimate of the marginal likelihood of dependent models of evolution, in which evolutionary transitions in lifestyle may depend upon reproductive mode and vice versa, consistently and strongly exceeds that of models of independent evolution (average BF = 14.14)(Table S4). The dependent mode of the reversible-jump MCMC analysis can visit both dependent and independent models of character evolution. Yet this analysis visited independent models less than 0.0001% of the time, which is lower than the 0.24% of visits to independent models predicted if independent and dependent models were equally likely. This further supports dependent, correlated evolution.

Evolutionary mechanisms underlying the correlation

We now test two hypotheses for the excess of outcrossing in animal parasites. First, uniparental reproduction may evolve more readily in free-living relative to animal parasitic lineages. Secondly, animal parasitism may evolve more readily in outcrossing relative to uniparental lineages. Both of these hypotheses are consistent with ancestral state reconstructions here and in prior studies suggesting parasitism and uniparental reproduction as derived states.

Our previous analysis (contrasting free-living and animal parasitic taxa) demonstrates significant support for dependent over independent models of evolution.

This allows for further investigation of the dependent models, specifically of the evolutionary transitions underlying correlated evolution. We therefore investigated the transition rate matrix of the dependent models to test our two hypotheses for the observed excess of outcrossing in animal parasites.

Hypothesis 1: uniparental reproduction evolves more frequently in free-living relative to animal parasitic lineages

This hypothesis predicts that the transition rate from outcrossing to uniparental reproduction is larger on a free-living (q_{13}) than on an animal parasitic (q_{24}) background (Fig. 2d). Comparison of transition rates under the dependent model of evolution demonstrates significant support for this hypothesis: q_{13} exceeds q_{24} 96.0% of the time by a large magnitude (average 16.17)(Fig. 2a). This result holds when the model is specified to prevent reversals from uniparental reproduction to outcrossing (Table S4). Moreover, the evolution of reproductive mode, rather than of lifestyle, determines the observed evolutionary patterns: transition rates for reproductive mode (q_{13} : outcrossing to uniparental) exceed those for lifestyle (q_{12} : free-living to animal parasitism) 96.1% of the time (average magnitude 15.57)(Fig. 2b). This finding is consistent with Hypothesis 1.

Hypothesis 2: animal parasitism evolves more frequently in outcrossing relative to uniparental lineages

This hypothesis predicts that the transition rate from free-living to animal parasitism is larger on an outcrossing (q_{12}) than on a uniparental (q_{34}) background. This hypothesis is

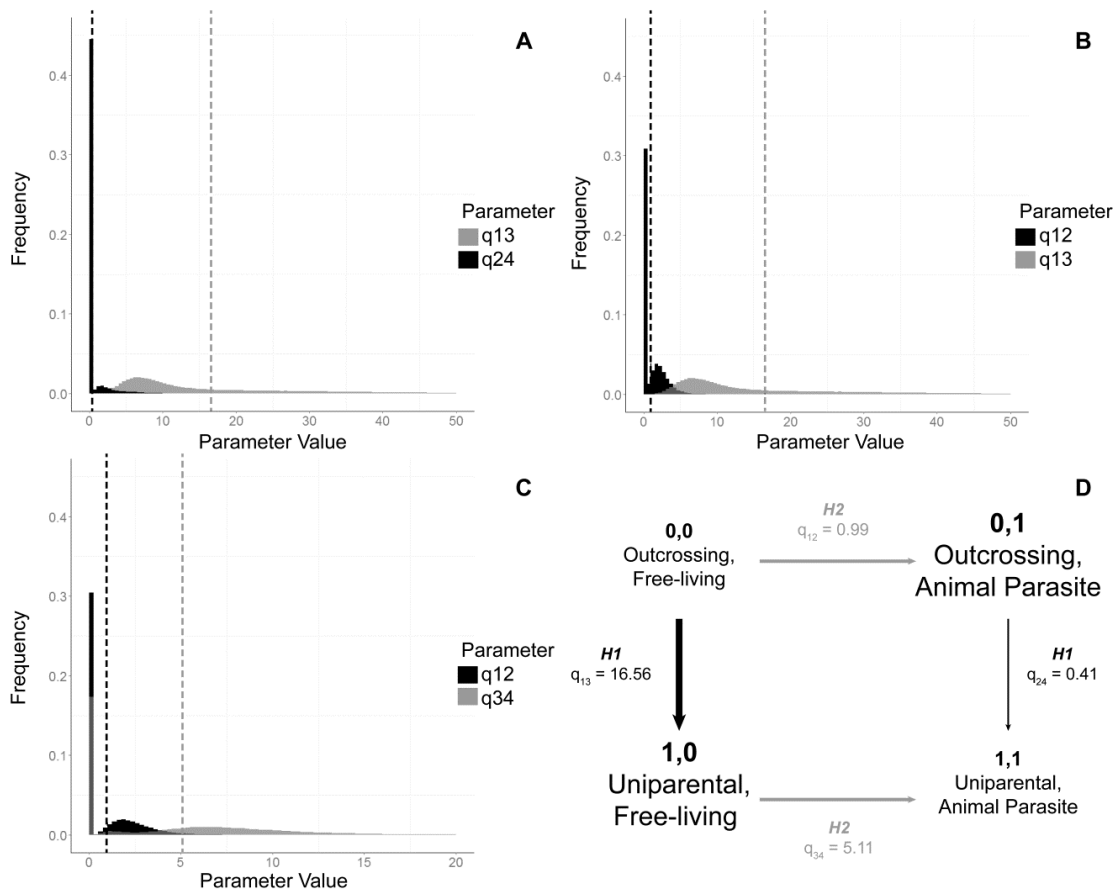


Figure 2: Estimated evolutionary transition rates in reproductive mode and lifestyle. (A-C) Posterior probability distribution of the values of transition rate parameters as estimated in one representative run of five dependent analyses in BayesDiscrete. Comparisons between two different transition rate distributions are displayed to test specific hypotheses. (A) H1: the transition rate from outcrossing to uniparental reproduction is greater on a free-living (q_{13}) than on an animal parasitic background (q_{24}). (B) H1: transition rates in reproductive mode (q_{13}) exceed those in lifestyle (q_{12}). (C) H2: the transition rate from free-living to animal parasitism is identical on outcrossing (q_{12}) and uniparental backgrounds (q_{34}). Dotted lines indicate the mean estimated transition rate for the corresponding parameter. (D) A diagram of investigated evolutionary transitions between the four different character states for reproductive mode and lifestyle. Larger type indicates character states for which a statistical excess of taxa is found. Line weight corresponds to the magnitude of the transition rate, estimated as the average value across five runs of the dependent analysis in BayesDiscrete. Black indicates transitions compared to test Hypothesis 1 and gray indicates Hypothesis 2.

not supported. Transition rates to animal parasitism on either background are low and statistically indistinguishable ($q_{12} > q_{34}$ 34.7% of the time on average) (Fig. 2c).

Discussion

In this study, we test the Red Queen Hypothesis's prediction that outcrossing should be more common in parasitic species than in their free-living relatives. We revisit Bell's (1982) investigation of the distribution of outcrossing and parasitism in the Nematode phylum using phylogenetic comparative tools. The results corroborate Bell's findings: there is a significant positive correlation between outcrossing and parasitism. Also consistent with Bell's results, we find that the relationship between outcrossing and parasitism is limited to animal parasites, with no correlation between plant parasitism and outcrossing. Accordingly, we tested hypotheses for the evolutionary mechanisms generating an excess of outcrossing in animal parasites. Our findings suggest that animal parasitic lineages are more resistant to invasion by uniparental strategies than are free-living lineages. These results strongly support the Red Queen Hypothesis as an explanation for the macroevolutionary distribution of outcrossing in the Nematoda.

Our phylogenetic findings corroborate prior studies of the Red Queen. Of most direct relevance is a theoretical study by Howard and Lively (2002) in which coevolution with hosts maintained outcrossing in parasites, though only in combination with mutation accumulation in clonal parasite lineages. Indirect support also comes from empirical studies of microbial experimental evolution. Coevolution of the bacteria *Bacillus thuringiensis* with nematode hosts resulted in bacterial populations with greater genetic diversity and more frequent horizontal gain of toxin genes, which are likely involved in

host interaction (Schulte et al. 2010; Schulte et al. 2013). The Red Queen Hypothesis has also been extended to rates of evolution, with the prediction that antagonistic coevolution leads to accelerated molecular evolution (Van Valen 1974; Hedrick 1994; Fischer and Schmid-Hempel 2005; Obbard et al. 2006). Paterson et al. (2010) demonstrated that, relative to phage evolution alone, coevolution of bacteriophage $\Phi 2$ with its host, resulted in significantly higher rates of molecular evolution for the phage, most notably at loci implicated in host interaction. These microevolutionary results support our macroevolutionary finding that the persistence of parasitic lineages requires forces that maintain genetic variation.

Interestingly, this finding is driven by animal parasites, with plant parasites showing no excess of outcrossing relative to free-living taxa. The occurrence of uniparental reproduction in nematode plant parasites has been previously noted (Triantaphyllou and Hirschmann 1964; Bell 1982; Castagnone-Sereno 2006; Castagnone-Sereno and Danchin 2014). We here propose hypotheses to explain this pattern. First, outcrossing in parasites could be maintained not by coevolution with hosts per se, but rather by coevolution with the adaptive immune systems of vertebrate hosts. Prior studies have not supported this hypothesis (Gemmil et al. 1997; Lythgoe 2000; West et al. 2001), excepting Galvani et al.'s (2003) theoretical demonstration that sexual populations of helminths can resist invasion by asexual mutants. In their study, the advantage of sexual populations stems from their ability to evade host immunity by maintaining strain diversity, which is stochastically lost in asexual parasite populations. Our results are also consistent with this hypothesis: the association of outcrossing and parasitism is present in animal parasites, the vast majority of which parasitize vertebrates, and absent in taxa

parasitizing plants. The animal parasites in our dataset that parasitize invertebrates are outcrossing but are too rare ($n=4$) to provide a valid contrast with vertebrate parasites. Additional sampling of taxa parasitizing invertebrates would allow for a test of the coevolving vertebrate immune system as a force maintaining outcrossing

Secondly, polyphagous, agricultural pests are over-represented among nematode plant parasites. Research in scale insects has demonstrated that asexual reproduction is more common in species that are polyphagous and/or pests (Ross et al. 2013). A broad host range may be linked with weak, non-specific coevolutionary interactions between hosts and parasites that fail to maintain outcrossing (Thompson 1999; Lajeunesse and Forbes 2002). This hypothesis predicts a larger host range for uniparental relative to outcrossing parasites. Alternately, Ross et al. (2013) attribute the relationship between polyphagy, pest status, and uniparental reproduction to population size. Large effective population sizes (N_e) of pest and/or polyphagous species may facilitate the persistence of uniparental lineages by reducing their probability of extinction by various forces (e.g. mutation accumulation, Hill-Robertson effects). Further research is required to properly contrast N_e of uniparental and outcrossing parasitic nematodes (Nadler 1995; Criscione and Blouin 2005).

Hypotheses based upon N_e present alternatives to the Red Queen Hypothesis (Muller 1964; Lynch et al. 1993; Otto and Barton 2001; Keightley and Otto 2006; Otto 2009; Hartfield et al. 2010; Hartfield et al. 2012), though these are not mutually exclusive (Howard and Lively 1994; Lively and Morran 2014). Indeed, prior theory on the maintenance of outcrossing in parasites argues for a combined role of host-parasite coevolution and forces that characterize finite populations (e.g. mutation accumulation

(Howard and Lively 2002), stochastic extinction (Galvani et al. 2003)). Other forces may also influence the distribution of outcrossing in the Nematoda. While the Red Queen offers an explanation for the short-term maintenance of outcrossing, limited adaptive potential and thus reduced diversification of uniparental lineages may contribute to outcrossing's long-term persistence (Fisher 1930; Muller 1932; Maynard Smith 1978; Nunney 1989; Goldberg et al. 2010; de Vienne et al. 2013). Selection for reproductive assurance has also been hypothesized to explain uniparental reproduction in taxa that inhabit unstable environments or disperse widely (e.g. androdioecy in free-living rhabditid nematodes) (Baker 1955; Pannell 2002; Weeks et al. 2006).

Our results are consistent with the prediction of the Red Queen, and thus we cannot falsify this major hypothesis for the maintenance of outcrossing. We do acknowledge three future improvements that would test the robustness of our results. First, current tests of correlated evolution cannot account for speciation and extinction rates, which may differ between reproductive modes (Fisher 1930; Muller 1932; Maynard Smith 1978; Nunney 1989; Goldberg et al. 2010). Ignoring this biological reality can result in over-estimation of reversals from uniparental reproduction to outcrossing (Maddison 2006; Goldberg and Igic 2008; Goldberg et al. 2010; Goldberg and Igic 2012). We rudimentarily addressed this issue by preventing this reversal in the Discrete analysis, and our results were qualitatively unchanged. Nonetheless, state-dependent diversification should be incorporated when improved phylogenies and comparative tools become available.

Secondly, Maddison and FitzJohn (2014) have recently argued that comparative methods for measuring correlations of discrete traits are flawed. When transitions in a

trait are rare or concentrated in single lineages, a fundamental assumption of these methods is violated. As a result, coincidence may be mistakenly interpreted as correlation. We find support for many dispersed transitions in both reproductive mode and lifestyle, suggesting that our dataset is relatively robust to Maddison and FitzJohn's (2014) methodological concerns. We cannot, however, reject the possibility that the observed associations are detected for reasons other than correlated evolution.

Thirdly, most nematode species remain undescribed (van Megen et al. 2009; Kiontke et al. 2011b). Prior studies of continuous traits suggest that under-sampling itself does not inflate estimates of phylogenetic correlation (Freckleton et al. 2002), but that biased sampling can (Ackerly 2000). Meldal et al. (2007) aimed to sample under-represented taxa for their phylogeny, but the sample of terrestrial taxa likely remains biased: first towards parasites, due to their relevance in public health and agriculture (Meldal et al. 2007; van Megen et al. 2009); secondly towards uniparental taxa, due to their tractability in the lab and over-representation in temperate regions where sampling has been concentrated (Bell 1982; Iqic and Kohn 2006; van Megen et al. 2009). Over-sampling of uniparental and/or parasitic taxa produces an under-representation of outcrossing, free-living taxa and thus potentially over-estimates the correlation of outcrossing and parasitism. We address this issue in the supplement via simulations to compare true measures of correlation to those obtained from biased sub-sampling. The simulation results argue that the evolutionary correlations reported here are unlikely to be an artifact of biased sampling of the Nematoda. Biased sampling can weakly inflate correlation estimates under stochastic character mapping but not under the Discrete method. Yet we find here that several unique tests support significant correlated

evolution of outcrossing and parasitism (d and m in stochastic character mapping, marginal likelihood and model visitation in BayesDiscrete).

The findings we present argue that the Nematoda is one of the most promising phyla in which to investigate the evolutionary and ecological forces underlying the maintenance of outcrossing. Moreover, the diversity of this group allows for an investigation of the mechanisms promoting genetic variation in parasite populations, a subject of the utmost importance (Grant 1994; Castagnone-Sereno 2002; Galvani et al. 2003; De Meeûs et al. 2009; Castagnone-Sereno and Danchin 2014). Until more complete phylogenies become available, such investigations should focus upon well-studied subgroups within the Nematoda. The genus of root-knot nematodes *Meloidogyne* presents an excellent opportunity to examine variation in reproductive mode within an obligately parasitic group (Castagnone-Sereno and Danchin 2014), while clades within the suborders Tylenchina and Rhabditina may be valuable for investigating transitions in lifestyle and reproductive mode at a finer scale (De Ley 2006).

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Supporting Information

The Supporting Information of the published article is available online at
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*From the works of Nathan A. Cobb,
"the father of U.S. nematology"*



Enoplus sp.: Original drawing made by W.E. Chambers for N.A. Cobb 1915: *Nematodes and their Relationships*, Figure 42, p. 484. Property of Nematology Investigations, USDA, Beltsville, MD. Image provided by Zafar Handoo.

Chapter 5

The evolution of reduced antagonism – a role for host-parasite
coevolution

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“Much of evolution is coevolution”

~ John N. Thompson, *The Geographic Mosaic of Coevolution* (2005), page

Abstract

Why do some host-parasite interactions become less antagonistic over evolutionary time? Vertical transmission can select for reduced antagonism. Vertical transmission also promotes coevolution between hosts and parasites. Therefore, we hypothesized that coevolution itself may underlie transitions to reduced antagonism. To test the coevolution hypothesis, we selected for reduced antagonism between the host *Caenorhabditis elegans* and its parasite *Serratia marcescens*. This parasite is horizontally transmitted, which allowed us to study coevolution independently of vertical transmission. After 20 generations, we observed a response to selection when coevolution was possible: reduced antagonism evolved in the co-passaged treatment. Reduced antagonism, however, did not evolve when hosts or parasites were independently selected, without coevolution. In addition, we found strong local adaptation for reduced antagonism between replicate host/parasite lines in the co-passaged treatment. Taken together, these results strongly suggest that coevolution was critical to the rapid evolution of reduced antagonism.

Introduction

Species interactions vary enormously, from highly antagonistic (e.g. host-parasite and predator-prey) to mutualistic. Moreover, the nature of a given interaction is not always evolutionarily stable: interspecific interactions can shift readily between parasitism and mutualism (e.g. Clay 1990; Herre 1993; Noda et al. 1997; Nishiguchi and Nair 2003; Sawada et al. 2003; Thompson 2005; Fenn and Blaxter 2006; Petersen and Tisa 2013) (reviewed in Thompson 1994b). The manner in which two partners interact may even vary within and between populations of the same species (Smith 1968; Thompson 1988;

Burdon et al. 1999; Kraaijeveld and Godfray 1999; Thompson and Cunningham 2002; Weeks et al. 2007). Of particular interest are those cases in which mutualisms seem to have arisen from parasitic interactions (e.g. Jeon 1972; Carroll 1988; Bandi et al. 1999; Hentschel et al. 2000; Dedeine et al. 2001; Dale et al. 2002; Weeks et al. 2007; Degnan et al. 2009; Hosokawa et al. 2010). These cases raise a puzzling, though pressing question: why, from an evolutionary standpoint, do transitions towards reduced antagonism occur?

Studies of parasite transmission mode have demonstrated that vertical transmission, from parent to offspring, can select for reduced antagonism (Bull et al. 1991; Herre 1993; Clayton and Tompkins 1994; Lipsitch et al. 1996; Turner et al. 1998; Messenger et al. 1999; Stewart et al. 2005; Sachs and Wilcox 2006). According to theory, the alignment of host and parasite fitness selects for reduced antagonism. Fitness alignment refers to a positive covariance of host and parasite fitness. Under vertical transmission, parasite fitness is contingent upon host survival and reproduction, and this positive fitness covariance favors reduced antagonism. Under horizontal transmission, the covariance of host and parasite fitness can be negative: selection for increased parasite transmission between hosts may select for increased within-host reproduction and thereby increased antagonism (Anderson and May 1982; Ewald 1987; Bull 1994; Frank 1996; Wade 2007). The direction of selection on antagonism thus varies with transmission mode.

The opportunity for coevolution also varies with transmission mode. Vertical transmission provides a unique opportunity for strong coevolution, because host and parasite lineages are paired over multiple generations. Conversely, horizontal transmission impedes tight coevolution between a single host and parasite lineage,

because the parasite lineage is continually transmitted between different host lineages. Therefore, in contrasting vertical with horizontal transmission, prior studies have not only compared experimental conditions with selection for and against antagonism. They have also inadvertently compared experimental conditions with high and low potential for coevolution, respectively.

To build upon this prior work, we tested the hypothesis that coevolution is fundamental to the evolution of reduced antagonism. We tested the role of coevolution, independently of vertical transmission, by using a horizontally transmitted parasite. This enabled us to impose selection for reduced antagonism directly, rather than indirectly through transmission mode. Accordingly, we did not manipulate transmission mode. Rather, we manipulated only the potential for coevolution in order to compare the degree of reduced antagonism achieved when coevolution was possible vs. that achieved when coevolution was prevented.

We tested this coevolution hypothesis through experimental evolution of the interaction between a nematode host, *Caenorhabditis elegans*, and a virulent bacterial parasite, *Serratia marcescens*. These experimental host and parasite populations were previously under selection for increased antagonism (Morran et al. 2011). In the present study, we reversed this selection. We favored reduced antagonism by selecting for hosts and parasites that were able to persistently interact, such that hosts survived to reproduction without clearing the parasites. In the co-passaged treatment, we allowed coevolution by selecting for reduced antagonism simultaneously in both partners. We contrasted this with the singly passaged treatments in which we prevented coevolution by selecting on one partner while holding the other constant. In all treatments, parasites were

transmitted horizontally, not vertically, in order to investigate coevolution independently of transmission mode.

If coevolution contributes to the reduction in antagonism between hosts and parasites, we predicted that the response to selection for reduced antagonism would be greater under co-passaging than under singly passaging. Our results support this prediction: reduced antagonism, in the form of a diminished fecundity cost of infection in hosts, evolved only in the pairing of co-passaged hosts and parasites. Moreover, shifts in host or parasite phenotypes alone could not explain the reduced antagonism of the co-passaged pairing. Rather, the interaction of host and parasite lineages was a significant factor in the reduction in antagonism. In addition, we found strong local adaptation between co-passaged host and parasite lineages. Our results argue that coevolution underlies the observed reduction in antagonism.

Methods

Host and parasite populations

Caenorhabditis elegans is a model for the study of host-parasite interactions (Kurz and Ewbank 2000), and there is reason to believe that *C. elegans* and *Serratia marcescens* interact in nature (Schulenburg et al. 2004; Schulenburg and Ewbank 2004; Pradel et al. 2007). This interaction is fascinating with respect to transitions in antagonism: *S. marcescens* is a virulent parasite not only of nematodes, but also of insects, corals, and humans (nosocomial). Yet some nematode species form mutualistic associations with *S. marcescens* and closely-related species (Petersen and Tisa 2013). The diversity of interactions between nematodes and *Serratia* argues that transitions in antagonism are

common. Our experimental evolution harnesses the evolutionary lability of this association to conduct a general test of the role that coevolution plays in transitions to reduced antagonism between naturally interacting species.

Replicate parasite populations were derived from *S. marcescens* strain Sm2170, which is highly virulent towards *C. elegans* (Schulenburg and Ewbank 2004). *Caenorhabditis elegans* hosts were derived from the strain PX382, an inbred line of CB4856. Five replicate populations were independently mutagenized with ethyl methanesulfonate to introduce genetic variation. These host populations were then co-passaged with populations of Sm2170 for 30 generations as part of a prior experiment (Morran et al. 2011). Assays of this experiment demonstrated that these host replicate lines adapted to resist their co-passaged parasite lines: co-passaged hosts showed significantly lower mortality rates than ancestral hosts when exposed to co-passaged parasites (Morran et al. 2011: mixed mating coevolved lines). Moreover, significant local adaptation of co-passaged parasite lines to kill sympatric co-passaged host lines strongly suggested genetic divergence between host lines under antagonistic coevolution (Morran et al. 2014: mixed mating coevolved lines).

We used these five divergent, co-passaged host populations as the ancestral replicate lines for our experimental evolution. We did so first because they provided an antagonistic starting point from which to select for reduced antagonism. Secondly, we observed a “reduced antagonism” phenotype at relatively high frequency in these host lines. *Serratia marcescens* typically colonizes the intestine of *C. elegans* hosts, resulting in loss of fecundity (Schulenburg and Ewbank 2004; Morran et al. 2011) and rapid host mortality (Mallo et al. 2002). In these lines, we observed hosts with light infections of *S.*

marcescens in their upper intestines. Through a stereoscope, the infection is readily evident as a bright red band or cluster of colonies just below the pharynx (Fig. S1). Preliminary observations indicated that hosts carrying *S. marcescens* in this region survive and reproduce without clearing the infection. Because host and parasite coexist for an extended period without a total loss of fitness for host (i.e. no death or sterilization) or parasite (i.e. no host recovery), we chose this “ruby-throated” phenotype as a model of reduced antagonism.

Prior to commencing experimental evolution, we quantified the frequency of the ruby-throated phenotype in a naïve nematode line vs. one with 30 generations of prior exposure to *S. marcescens*. 750 L3-L4 nematodes were added to a lawn of Sm2170 (as in *Serratia* selection plates, described below). Sixty hours later, we counted the number of ruby-throated hermaphrodites on the plates. We assayed ten plates for each of the two host lines. To compare ruby-throated frequency between lines, we performed a Student’s t-test in R v3.0.2 (R Core Team 2013)

Experimental evolution of reduced antagonism

We devised experimental evolution treatments in order to select for 1. Ruby-throated hosts, which survive and reproduce with a persistent infection and/or 2. Ruby-throated parasites, which establish persistent infections without rapidly killing or sterilizing the host. Treatments selected for the evolution of reduced antagonism under conditions that did or did not permit coevolution (Fig. 1). In the “co-passaged” treatment, coevolution of host and parasite was possible: hosts and parasites were simultaneously passaged. Two “singly-passaged” treatments did not permit coevolution: selection for reduced

antagonism was conducted separately on host and parasite. Each generation of selection, ruby-throated hosts and parasites were paired with static ancestral parasites and hosts, respectively. Two control treatments prevented coevolution and symbiosis: control hosts and parasites were selected at random and passaged in the absence of live parasites and hosts, respectively. Control treatments served to account for genetic drift and non-focal selection pressures. Each of these five treatments had five replicate host populations; these were the five divergent lineages resulting from a prior experimental evolution project, as discussed above. We conducted 20 generations of selection.

Transfer and Selection on SSPs

We describe here the details of the co-passaged treatment, followed by specific modifications for the four remaining treatments (Fig. 1). Selection was performed on *Serratia* selection plates (SSPs), which we constructed as in Morran et al. (2011). We added 1500 L3-L4 nematodes to a lawn of *S. marcescens* to force hosts and parasites to interact. Hosts migrated towards the opposite half of the plate, seeded with a lawn of OP50 (food source). After 60 hours on the plates, we transferred approximately 20 ruby-throated hosts per replicate to a new plate, seeded with only OP50. Ruby-throated hosts were then allowed to reproduce. After 48 hours, parental ruby-throated hosts were separated from their F1 offspring by size and phenotype. F1 host offspring were washed with M9 buffer and transferred to a plate seeded with OP50 to reproduce for 65 hours. Ruby-throated parasites were extracted from the ~20 parental ruby-throated hosts by washing hosts repeatedly with M9 buffer to remove external bacteria and then crushing them to release the ruby-throated bacteria. The isolated bacteria were grown on a plate

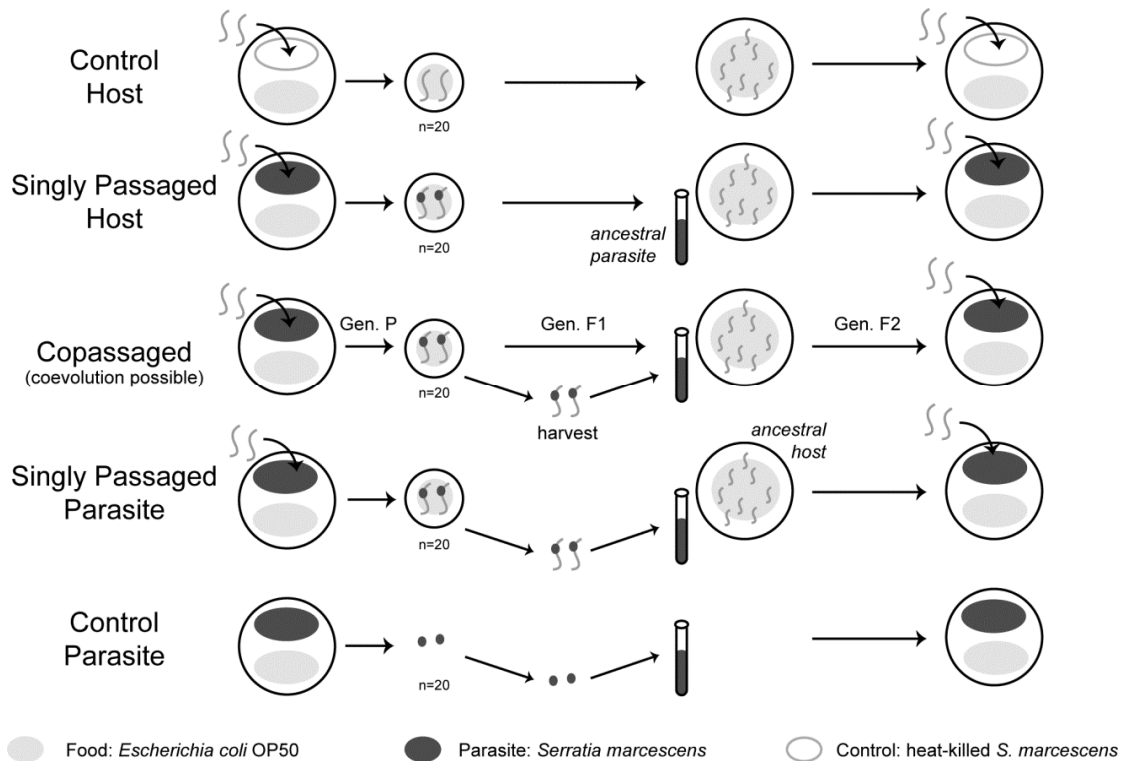


Figure 1: Experimental evolution design. Five experimental evolution treatments were established to evaluate the role of coevolution in the evolution of host-parasite antagonism. Selection for the ruby-throated phenotype was performed on *Serratia* Selection Plates (SSPs). Twenty ruby-throated adults were then transferred to new plates to reproduce. In the co-passaged treatment, in which coevolution was possible (center row), both the F1 ruby-throated hosts and parasites were propagated for an additional generation. F2 hosts and parasites were then combined on a new SSP for the next generation of selection. In the singly-passaged treatments, ruby-throated hosts (2nd row) and parasites (4th row) were passaged with static ancestral parasite and host populations, respectively. In the control treatments, healthy hosts (1st row) and free-living parasites (5th row) were passaged in the absence of live parasites or hosts, respectively.

for 24 hours at 28°C. Colonies were then randomly selected for growth in Luria Broth (LB) overnight at 28°C (n=10). This culture was used to seed a new SSP. 1500 L3-L4 F2 hosts were added to this plate for the next generation of selection.

In the singly-passaged host treatment, ruby-throated hosts were passaged as in the co-passaged treatment. However, in place of the ruby-throated parasites, we used ancestral Sm2170 for seeding of the subsequent SSP. In the singly-passaged parasite treatment, ruby-throated parasites were passaged as in the co-passaged treatment. However, each generation, in place of the ruby-throated hosts, we obtained offspring from ancestral host populations maintained at 15°C. In the control host treatment, SSPs were constructed with heat-killed Sm2170 in place of live parasites. Twenty hosts were selected at random from SSPs and allowed to reproduce. In the control parasite treatment, SSPs were constructed as in the co-passaged treatment, but no hosts were added. Free-living parasites were passaged by randomly selecting 20 colonies from the Sm2170 lawn in order to roughly mimic the number of parasites passaged when transferring 20 ruby-throated hosts. Host and parasite lines were stored at -80°C after 20 generations of selection (Supplemental Methods).

Assays of Frequency, Fecundity and Virulence

Both an increase in frequency of the ruby-throated phenotype and an increase in fecundity of ruby-throated hosts are consistent with a response to selection for reduced antagonism. We therefore compared ruby-throated frequency and fecundity in various combinations of host and parasite lines in order to 1. Test if reduced antagonism evolved primarily when coevolution was possible (co-passaged treatment) (Table 1A,B) and 2. Evaluate the relative contributions of host vs. parasite evolution in the transition to reduced antagonism (Table 1C,D). We reduced variation resulting from sex-specific differences in the ruby-throated phenotype by selecting 20 unmated hermaphrodites to

establish low-male subpopulations. Assays were then performed so as to replicate the conditions of experimental evolution.

We first quantified changes from the ancestor in the frequency of formation of the ruby-throated phenotype. Two hundred L3-L4 nematodes from low-male lines were added to SSPs, constructed as outlined above. After 65 hours, we counted the total numbers of adult hermaphrodites and adult ruby-throated hermaphrodites on the OP50 halves of the SSPs. We assayed three replicates for each combination of host and parasite line (combinations given in Table 1A, C-D). For all assays, host lines were paired with sympatric parasite lines except in the local adaptation assays below. For each comparison, a separate ANOVA was performed in SPSS v21 (IBM) to test the effect of treatment, line, replicate, and the treatment by line interaction on the frequency of ruby-throated hosts. Replicate was excluded when insignificant. In all statistical analyses, treatment was a fixed effect, while line and replicate were treated as random. We additionally performed a linear contrast test of ruby-throated frequency in control, singly-passaged, and co-passaged pairings vs. ancestral pairings.

Fecundity assays were an extension of the frequency assays above. After measuring ruby-throated frequency, we selected ten ruby-throated hermaphrodites from each replicate and transferred them to individual plates. In total, we isolated 30 ruby-throated hermaphrodites (10 x 3 replicates) per combination of host and parasite line. After 48 hours, we counted the number of offspring per hermaphrodite. For each comparison (Table 1A-D), a separate ANOVA was performed on ruby-throated fecundity, as described above. We similarly measured the fecundity of uninfected hosts

Table 1: Pairings of host and parasite populations for assays of ruby-throated frequency and fecundity

<i>A. Interaction: experimental host and parasite populations combined¹</i>		<i>B. Interaction: host and parasite populations paired as in experimental selection²</i>	
Host	Parasite	Host	Parasite
Ancestor	Ancestor (Sm2170)	Singly passaged	Ancestor
Control	Control	Ancestor	Singly passaged
Singly passaged	Singly passaged	Copassaged	Copassaged
Copassaged	Copassaged		
<i>C. Host alone: experimental host populations with ancestral parasites³</i>		<i>D. Parasite alone: experimental parasite populations with ancestral hosts⁴</i>	
Host	Parasite	Host	Parasite
Control	Ancestor	Ancestor	Control
Singly passaged	Ancestor	Ancestor	Singly passaged
Copassaged	Ancestor	Ancestor	Copassaged

¹Compares host and parasite lines paired according to shared evolutionary history. If coevolution contributes to reduced antagonism, the response to selection for reduced antagonism will be greatest in the copassaged pairing.

²Compares pairings that were selected upon during experimental evolution to further test the prediction that the response to selection for reduced antagonism will be greatest in the copassaged pairing.

³Compares changes in passaged host lines independent of the parasite to test if the reduced antagonism observed in the copassaged pairing can be attributed to evolution of copassaged host populations alone.

⁴Compares changes in passaged parasite lines independent of the host to test if the reduced antagonism observed in the copassaged pairing can be attributed to evolution of copassaged parasite populations alone.

from all treatments in order to evaluate the contribution of host evolution alone to observed reductions in antagonism (Supplemental Methods).

We also quantified the change in parasite virulence during experimental evolution in order to evaluate the contribution of parasite evolution alone to observed reductions in antagonism. Parasite virulence was assessed through mortality assays, as described in Morran et al. (2011). We measured the mortality rate as the proportion of dead/morbid hosts after 24 hours of exposure of standard host lines to ancestral, control, singly-passaged, and co-passaged parasite lines (Supplemental Methods). An ANOVA was performed as described above.

Local adaptation

To determine if co-passaged hosts and parasites coevolved during experimental evolution, we tested the degree of local adaptation for reduced antagonism. We performed fully reciprocal cross-infections between our five co-passaged host and parasite lines (25 combinations). Fecundity was measured as described above, excepting that for each combination, 15 adult ruby-throated hermaphrodites were isolated from a single SSP. Analyses of local adaptation were based upon Morran et al. (2014). We first performed an ANOVA to test for a significant interaction effect of host and parasite line on ruby-throated fecundity. We then evaluated overall local adaptation by performing a linear contrast test of the fecundity of ruby-throated hosts in all sympatric pairings (n=5) versus all allopatric pairings (n=20) (Blanquart et al. 2013).

We also performed fine-scale local adaptation tests for each host and parasite pairing (Morran et al. 2014). We did so using a linear contrast test of the sympatric host-

parasite pairing against all allopatric pairings: we compared the fecundity of the sympatric pairing (e.g. co-passaged host line 1 with co-passaged parasite line 1) with that of the host population plus allopatric parasite populations (e.g. co-passaged host line 1 with co-passaged parasite lines 2-5) and that of the parasite population plus allopatric host populations (e.g. co-passaged parasite line 1 with co-passaged host lines 2-5). One-tailed linear contrast tests were performed independently for lines 1-5 to evaluate the hypothesis that the fine-scale tests of local adaptation reflected the overall test of local adaptation. We report the results of tests for which equal variances were not assumed, as variances were significantly different for three of the six tests performed.

Results

Reduced antagonism is only observed under co-passaging

After 20 generations of selection, we quantified the response to selection for reduced antagonism by measuring the frequency and fecundity of ruby-throated hosts. We compared these traits in the following pairings: ancestral hosts and parasites; control hosts and parasites; singly-passaged hosts and parasites; co-passaged hosts and parasites (Table 1A). If coevolution contributes to reduced antagonism, we predicted that the co-passaged pairing would show the greatest response to selection for reduced antagonism.

Increased frequency of the ruby-throated phenotype is consistent with reduced antagonism, because host and parasite engage more frequently in persistent, non-lethal interactions. The frequency of the ruby-throated phenotype did not differ between host-parasite combinations (Table S1A: $F_{(3,12)}=0.938$, $p=0.453$). Specifically, the frequency did not increase in evolved relative to ancestral pairings (linear contrast: $p=0.254$, one-

tailed)(Fig. S2A, *bar* $a \approx b, c, d$). Thus experimental selection did not alter rates of persistent association between host and parasite.

Reduced antagonism was observed when measured as changes in fecundity over the course of the experiment. Increased fecundity of ruby-throated hosts is consistent with reduced antagonism, because the fitness cost of an active infection is reduced. The fecundity of ruby-throated hosts differed between host-parasite combinations (Table S2A: $F_{(3,12)}=4.643$, $p=0.022$)(Fig. 2A): ruby-throated hosts from co-passaged pairings displayed significantly elevated fecundity relative to ancestral ($p=0.015$, *bar* $d > a$), control ($p<0.001$, $d > b$), and singly-passaged ($p<0.001$, $d > c$) pairings. Control and singly-passaged pairings did not differ from the ancestral pairing ($p=0.481$, $a \approx b$; $p=0.521$, $a \approx c$, respectively), nor from one another ($p=1.00$, $b \approx c$). Thus, among these pairings, a response to selection for reduced antagonism only occurred in the host-parasite pairs that had the potential for coevolution.

However, singly-passaged hosts and parasites may have evolved reduced antagonism specifically in combination with the parasite and host populations, respectively, that they encountered during the experiment. Therefore, we also compared ruby-throated fecundity in the pairings under selection in experimental evolution: singly-passaged hosts with ancestral parasites, ancestral hosts with singly-passaged parasites, and co-passaged hosts with co-passaged parasites (Table 1B). The fecundity of ruby-throated hosts again differed significantly (Table S2B: $F_{(2,8)}=5.073$, $p=0.038$), with the co-passaged pairing having significantly higher fecundity than either singly-passaged pairing (host: $p=0.014$, *bar* $d > f$; parasite: $p=0.013$, $d > i$)(Fig. 2A-C). Furthermore, singly-

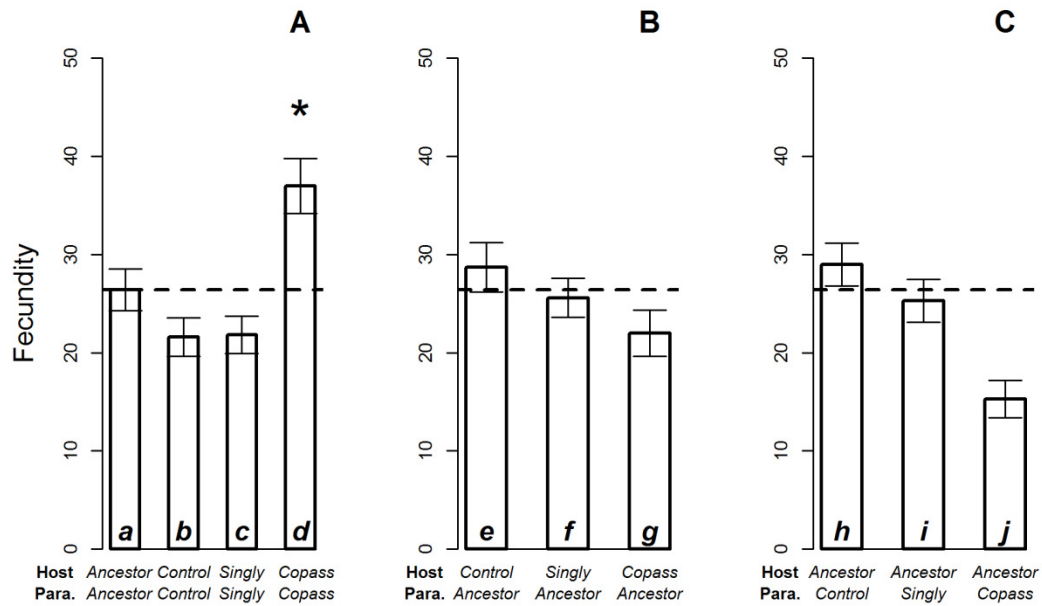


Figure 2: Fecundity of ruby-throated hosts. The fecundity of ruby-throated hosts across different pairings of host and parasite populations. (A) Host and parasite pairings according to shared evolution history. The fecundity of co-passaged pairings was significantly elevated relative to ancestral, control, and singly-passaged pairings. (B) Hosts paired with ancestral parasites. Ruby-throated fecundity did not differ when host populations were paired with the same (ancestral) parasite population. (C) Parasites paired with ancestral hosts. Ruby-throated fecundity differed marginally when parasite populations were paired with the same (ancestral) host populations. The dashed line marks the starting point of experimental evolution: mean ruby-throated fecundity resulting from pairing ancestral hosts with ancestral parasites. Each bar is an average of fecundity counts obtained from 150 ruby-throated hermaphrodites (10 hermaphrodites per replicate assay plate, three replicates per each of five lines), and error bars give the standard error of mean fecundity counts. Individual bars are referred to by letter in the text.

passed host and parasite populations did not differ in their response to selection: ruby-throated fecundity of singly-passaged hosts with ancestral parasites did not differ from singly-passaged parasites with ancestral hosts ($p=0.999, f \approx i$). Therefore, reduced antagonism evolved only when coevolution was possible.

Host evolution alone cannot explain reduced antagonism

We only observed reduced antagonism in the co-passaged pairing, arguing for a role for coevolution. However, the case for coevolution would be significantly weakened if traits in the co-passaged host or parasite populations alone could explain the reduction in antagonism. We first tested if co-passaged host populations alone could recapitulate the increase in ruby-throated fecundity of the co-passaged pairing. To do so, we asked if the fecundity of co-passaged hosts exceeded that of control and singly-passaged hosts when all were paired with ancestral parasites (Table 1C; Fig. 2B, *bar g vs. e,f*). The fecundity of ruby-throated hosts did not differ between any pairings with ancestral parasites (Table S2C: $F_{(3,12.31)}=0.142, p=0.933$). Therefore evolution of co-passaged hosts alone cannot explain reduced antagonism in the co-passaged pairing.

Moreover, a general increase in the fecundity of healthy co-passaged hosts cannot explain the elevated ruby-throated fecundity. We observed no significant difference in the fecundity of uninfected hosts from the ancestral, control, singly-passaged, and co-passaged populations (Table S3: $F_{(2,8)}=3.160, p=0.097$)(Fig. S3).

Parasite evolution alone cannot explain reduced antagonism

We then tested if the co-passaged parasite populations alone could recapitulate the increase in ruby-throated fecundity of the co-passaged pair. To do so, we asked if the fecundity of ancestral hosts infected with co-passaged parasites exceeded that of ancestral hosts infected with control and singly-passaged parasites (Table 1D; Fig. 2C, *bar j* vs. *h,i*). The difference in fecundity of ruby-throated hosts from these pairings was marginally significant (Table S2D: $F_{(3,12)}=2.784$, $p=0.086$) due to the relatively *depressed* fecundity of co-passaged parasites with ancestral hosts. This result strongly argues that the evolution of co-passaged parasites alone cannot explain reduced antagonism in the co-passaged pairing.

Moreover, decreased virulence of the co-passaged parasite populations cannot explain the elevated ruby-throated fecundity. We measured the mortality rate of ancestral hosts exposed to ancestral, control, singly-passaged, and co-passaged parasites. Mortality rate differed significantly between parasite treatment (Table S4: $F_{(3,12)}=14.370$, $p<0.001$)(Fig. S4): ancestral parasites induced a significantly higher mortality rate than all other parasites ($p<0.001$ for each). Mortality rate with co-passaged parasites was low but equivalent to that of control ($p=0.407$) and singly-passaged ($p=0.878$) parasites. Parasite virulence declined uniformly across all experimental treatments and thus cannot explain reduced antagonism in the co-passaged pair.

Strong local adaptation is evidence for coevolution

Our results indicate that coevolution was critical to the response to selection for reduced antagonism. However, the degree to which co-passaged hosts and parasites coevolved

was uncertain. Tests of local adaptation are commonly used to detect reciprocal adaptation. We measured ruby-throated fecundity in fully reciprocal cross-infections among our five co-passaged host and parasite populations. Consistent with local adaptation, we found a significant interaction effect of co-passaged host and parasite population (line) on ruby-throated fecundity (Table S5A: $F_{(16,298)}=3.560$, $p<0.001$)(Fig. 3).

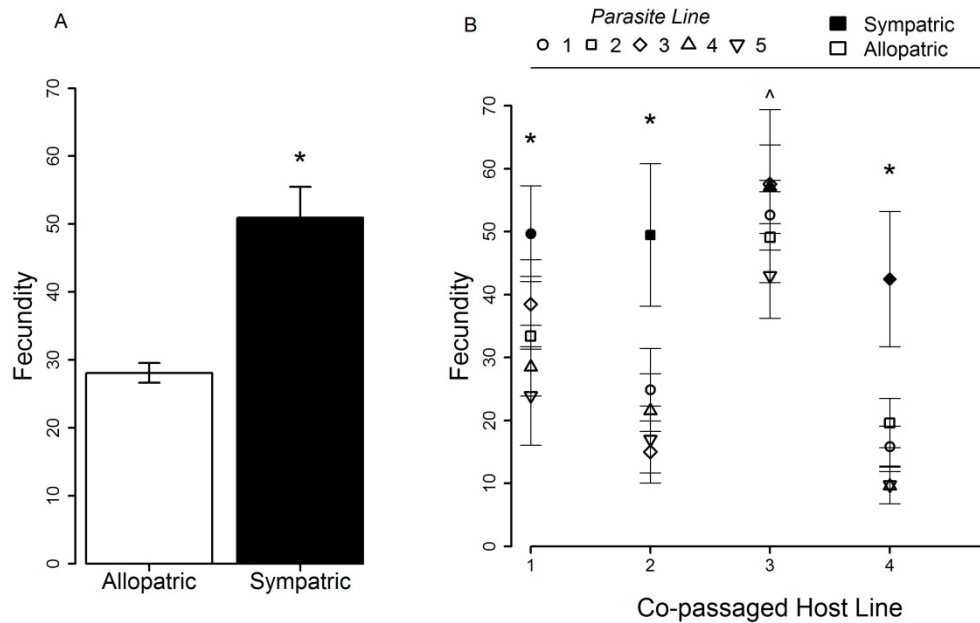


Figure 3: Local adaptation of co-passaged populations. Reciprocal cross-infections of co-passaged host and parasite lines were performed to test for local adaptation. (A) Sympatric co-passaged pairings displayed significantly higher fecundity than allopatric pairings. The allopatric bar is an average of fecundity counts obtained from 300 hermaphrodites (15 hermaphrodites from each of 20 allopatric combinations), and the sympatric bar is an average of 75 hermaphrodites (5 sympatric combinations). (B) Each co-passaged host-parasite pairing supports the results of the overall analysis: ruby-throated fecundity was significantly higher in sympatric relative to allopatric pairings for lines 1,2,4, and 5 (*) and marginally significantly for line 3 (^). Each point is an average of fecundity counts obtained from 15 hermaphrodites, and all error bars give the standard error of mean fecundity counts.

Given this significant interaction, we tested the hypothesis that ruby-throated fecundity in sympatric pairings exceeds that in allopatric pairings. We first made the comparison across all co-passaged host and parasite lines. Consistent with local adaptation, we found that the ruby-throated fecundity of sympatric pairings significantly exceeded that of allopatric pairings (Table S5B: $p < 0.001$)(Fig. 3A).

We then made the comparison for each replicate population within the co-passaged treatment by contrasting each sympatric host-parasite pairing with its eight possible allopatric pairings (i.e. the focal host population paired with the four allopatric parasite populations and the focal parasite population paired with the four allopatric host populations). Consistent with local adaptation, the fecundity of the sympatric pairing exceeded that of allopatric pairings to a significant degree for host and parasite lines 1,2,4 and 5 and marginally for host and parasite lines 3 (Table S5B: 1: $p = 0.017$; 2: $p = 0.027$; 3: $p = 0.059$; 4: $p = 0.046$; 5: $p < 0.001$; one-tailed tests)(Fig. 3B). These results suggest rapid divergence among replicate populations in the co-passaged treatment, consistent with coevolution of co-passaged host and parasite populations.

Discussion

In this study, we tested the hypothesis that coevolution can contribute to the evolution of reduced antagonism between hosts and parasites. We selected directly on a phenotypic indicator of reduced antagonism under conditions that either did or did not permit coevolution. Our results strongly support the coevolution hypothesis: after 20 generations of selection, reduced antagonism only evolved in response to selection when coevolution was possible (Fig. 2A).

We found no support for the idea that reduced antagonism arose from the evolution of host or parasite traits alone (Fig. 2B,C). Co-passaged host or parasite lineages, when paired with ancestral parasites or hosts, respectively, did not show reduced antagonism. We particularly note that declines in parasite virulence alone were insufficient for reduced antagonism. The virulence of the co-passaged parasite lineages towards ancestral hosts was no lower than that of singly-passaged and control parasite lineages: virulence declined in all experimental and control parasite lineages (Fig. S4). A possible explanation for this is that the free-living generations during experimental evolution of the parasite lines (growth in LB or on agar plates) exerted negative or relaxed selection on virulence or correlated traits (Caraco and Wang 2008; Friman et al. 2009; Mikonranta et al. 2012; Wasik et al. 2015). Regardless of the mechanism, differential evolution of virulence in our experimental lines cannot explain the reduced antagonism observed in the co-passaged pairing.

Our results argue that the evolution of reduced antagonism required coevolution, which may result in local adaptation (Parker 1985; Lively 1989). We found strong evidence of local adaptation of co-passaged host and parasite lineages for reduced antagonism (Fig. 3), further demonstrating that co-passaged host and parasite lineages did indeed coevolve. These results also demonstrate that adaptation of co-passaged host and parasite replicate lines occurred rapidly, within 20 generations of selection. This is particularly noteworthy given that genetic drift likely reduced the fixation probability of beneficial mutations in our experiment (Gillespie 1998). Thus, our results may have been even stronger with larger effective population sizes. Finally, local adaptation indicates that replicate pairs diverged rapidly, arriving at reduced antagonism via distinct

evolutionary routes. We did use genetically distinct ancestral populations to establish our five replicate host lines, which may explain why we see such a strong signal of divergence between our pairings. However, the use of distinct ancestral host lineages makes the repeated evolution of reduced antagonism in the co-passaged treatment even more striking.

Taken together, our findings argue that reduced antagonism arises from the interaction of reciprocally adapting host and parasite lineages. We therefore propose that coevolution may contribute to the evolution of reduced antagonism when selection is imposed by fitness alignment under vertical transmission (positive fitness covariance) (Bull et al. 1991; Herre 1993; Clayton and Tompkins 1994; Lipsitch et al. 1996; Turner et al. 1998; Messenger et al. 1999; Stewart et al. 2005; Sachs and Wilcox 2006). Our findings also show that reduced antagonism can evolve without vertical transmission if selection is directly imposed and coevolution is present. It is unlikely that we imposed any indirect selection via fitness alignment, because the parasite is horizontally transmitted. Moreover, parasite fitness depended heavily upon reproduction during the “free-living” phase: multiple free-living generations alternated with selection events during infection. Similar transmission modes are common in nature, including in mutualisms of *Vibrio* bacteria with squid and rhizobia with legumes (as reviewed in Bright and Bulgheresi 2010).

In addition, fitness alignment should correspond to an increase in the frequency of the ruby-throated phenotype, which we did not observe. When a parasite genotype establishes the ruby-throated phenotype with minimal cost to its host, that host genotype will comprise a larger proportion of the next host generation. Assuming genetic

specificity of ruby-throated host and parasite, those parasite offspring will then have more opportunities for host establishment. The frequency of the ruby-throated phenotype should accordingly increase. The fact that we failed to observe any increase in frequency argues against fitness alignment in our study. The lack of response in ruby-throated frequency in our study may alternately be due to limited additive genetic variance for this trait following prior evolution of these host populations (Morran et al. 2011).

Previous studies of fitness alignment have demonstrated that reduced antagonism arises from an interaction of host and parasite genotypes, which hints at the significance of coevolution (Traub 1939; Bull et al. 1991; Bull and Molineux 1992). After selection under vertical transmission of bacteriophage f1 with *Escherichia coli*, Bull and Molineux (1992) observed increases in the growth rate of infected host populations and in phage-mediated protection against infection by alternate phages. For most experimental lines, growth rate and protection were greater when phage were paired with their co-passaged hosts than with the ancestor. Several studies, however, present contradictory results: evolution of host (Helling et al. 1981; Bouma and Lenski 1988) or parasite traits alone (Jeon 1972; Sachs and Wilcox 2006; Weeks et al. 2007; Jansen et al. 2015) could explain the observed reduction in antagonism. Importantly, these studies all examined the endpoint of long-term selection under vertical transmission, rather than directly testing the role of coevolution.

Here, we directly tested the contribution of coevolution under horizontal transmission. Our experimental coevolution design is particularly powerful in contrasting coevolution with independent evolution (Brockhurst and Koskella 2013) and could be applied to many other experimental symbiosis models in which partners can be

dissociated (Denison et al. 2003; e.g. Stewart et al. 2005; Sachs and Wilcox 2006; Hillesland and Stahl 2010; Jansen et al. 2015). Overall, we find that coevolution is critical to the evolution of reduced antagonism. Similar investigations of natural symbioses are required to determine if coevolution is generally a factor in evolutionary transitions towards reduced antagonism. Further support for the significance of host-parasite coevolution would argue for its inclusion in models of virulence evolution, which primarily focus upon parasite evolution alone (though see van Baalen 1998; Restif et al. 2001; Gandon et al. 2002; Day and Burns 2003; Restif and Koella 2003; Little et al. 2010).

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Supporting Information

Thawing experimental lines

Following 20 generations of selection, host and parasite lines were stored at -80°C until use in assays. Assays were performed to quantify changes in the frequency of the ruby-

throated phenotype, fecundity of ruby-throated and uninfected hosts, virulence of parasite populations, and local adaptation within the co-passaged treatment. Prior to use in assays, the required host lines were thawed and cycled at room temperature for two to four generations to recover. Thawed parasite populations were grown overnight on plates at 28°C to obtain individual colonies, one of which was then selected for growth in LB overnight at 28°C. These cultures were used to seed SSPs or mortality plates for use in assays.

Uninfected host fecundity

We measured the fecundity of uninfected hosts from all treatments in order to evaluate the contribution of host evolution alone to observed reductions in antagonism. Specifically, we evaluated the extent of the shift towards delayed reproduction relative to ancestral hosts. Our experimental evolution imposed selection for delayed reproduction: only offspring produced ~48 hours into adulthood were passaged to the next generation. If a shift towards delayed reproduction occurred exclusively for co-passaged host populations, we could not conclude that the elevated fecundity of the co-passaged pairing is a result of the host-parasite interaction itself. We therefore measured the fecundity of uninfected hosts, under the same timing as that of measures of ruby-throated fecundity and of selection during experimental evolution. SSPs were constructed as above, though lacking the *S. marcescens* lawn: hosts were not exposed to parasites. Two hundred nematodes of each ancestral, control, singly-passaged, and co-passaged line were added to one SSP each. Sixty hours later, we randomly selected 15 adult hermaphrodites and transferred them to individual plates. After 48 hours, we counted the number of offspring

produced. An ANOVA was performed to test the effect of treatment and line on uninfected host fecundity.

Parasite virulence

We also quantified the change in parasite virulence during experimental evolution in order to evaluate the contribution of parasite evolution alone to observed reductions in antagonism. Parasite virulence was assessed through mortality assays, as described in Morran et al. (2011): higher mortality rates are consistent with higher parasite virulence.

We measured the mortality rate of standard host lines (ancestral host lines 1-5) when paired with ancestral parasite Sm2170 and passaged parasite lines (control parasite 1-5, singly-passaged parasite 1-5, and co-passaged parasite 1-5). Four plates per parasite line were seeded with a ~ 6 cm lawn of 40 μ L of *S. marcescens*. Two hundred nematodes from ancestral lines were added to parasite lawns of their corresponding line. We counted the number of dead and morbid nematodes 24 hours later. Mortality rate was calculated as the number of dead/morbid nematodes divided by the total worms added to the plate. An ANOVA was performed to test the effect of parasite treatment, line, replicate, and the interaction of treatment and line on host mortality rate.

Supplemental Tables

Table S1: Results of Analysis of Variance for ruby-throated frequency

A. Interaction: experimental host and parasite populations combined

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Treatment</i>	3	0.010	0.938	0.453
<i>Line</i>	4	0.110	10.063	0.001
<i>Interaction</i>	12	0.010	3.884	0.001
<i>Error</i>	40	0.003		

B. Host alone: experimental host populations with ancestral parasites

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Treatment</i>	3	0.030	4.420	0.026
<i>Line</i>	4	0.040	5.352	0.0100
<i>Interaction</i>	12	0.007	4.517	<0.001
<i>Error</i>	40	0.002		

C. Parasite alone: experimental parasite populations with ancestral hosts

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Treatment</i>	3	0.070	3.080	0.068
<i>Line</i>	4	0.040	2.047	0.151
<i>Interaction</i>	12	0.020	14.785	<0.001
<i>Error</i>	40	0.001		

Table S2: Results of Analysis of Variance for ruby-throated fecundity*A. Interaction: experimental host and parasite populations combined*

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Treatment</i>	3	775.89	4.643	0.022
<i>Line</i>	4	504.96	3.021	0.061
<i>Interaction</i>	12	167.13	2.009	0.049
<i>Error</i>	40	83.17		

B. Interaction: host and parasite populations paired as in experimental selection

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Replicate</i>	2	373.21	3.551	0.042
<i>Treatment</i>	2	645.89	5.073	0.038
<i>Line</i>	4	193.13	1.517	0.285
<i>Interaction</i>	8	127.32	1.212	0.328
<i>Error</i>	28	105.09		

C. Host alone: experimental host populations with ancestral parasites

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Replicate</i>	3	321.75	7.702	<0.001
<i>Treatment</i>	3	51.05	0.142	0.933
<i>Line</i>	4	111.05	0.286	0.882
<i>Interaction</i>	12	398.21	9.532	<0.001
<i>Error</i>	37	41.78		

D. Parasite alone: experimental parasite populations with ancestral hosts

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Treatment</i>	3	533.63	2.784	0.086
<i>Line</i>	4	301.74	1.574	0.244
<i>Interaction</i>	12	191.65	2.962	0.005
<i>Error</i>	40	64.70		

Table S3: Results of Analysis of Variance for fecundity of healthy hosts

Uninfected: experimental host populations alone

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Treatment</i>	2	1471.92	3.160	0.097
<i>Line</i>	4	182.39	0.392	0.809
<i>Error</i>	8	465.74		

Table S4: Results of Analysis of Variance for host mortality

Experimental parasite populations with ancestral hosts

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Replicate</i>	3	0.010	2.428	0.075
<i>Treatment</i>	3	0.110	14.370	<0.001
<i>Line</i>	4	0.050	6.149	0.006
<i>Interaction</i>	12	0.008	1.796	0.071
<i>Error</i>	57	0.004		

Table S5: Results of Analysis of Variance and linear contrasts for local adaptation*A. Analysis of Variance: co-passaged host and parasite line as predictors of ruby-throated fecundity*

	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Host</i>	4	9251.60	4.384	0.014
<i>Parasite</i>	4	412.57	0.196	0.937
<i>Interaction</i>	16	2133.01	3.560	<0.001
<i>Error</i>	298	599.24		

B. Linear contrasts: sympatric vs. allopatric pairings

	<i>T</i>	<i>p</i>	<i>Hypothesis</i>
Overall	4.742	<0.001	Two-tailed
<i>Line 1</i>	2.363	0.017	One-tailed
<i>Line 2</i>	2.096	0.027	One-tailed
<i>Line 3</i>	1.684	0.059	One-tailed
<i>Line 4</i>	1.828	0.046	One-tailed
<i>Line 5</i>	4.447	<0.001	One-tailed

Supplemental Figures

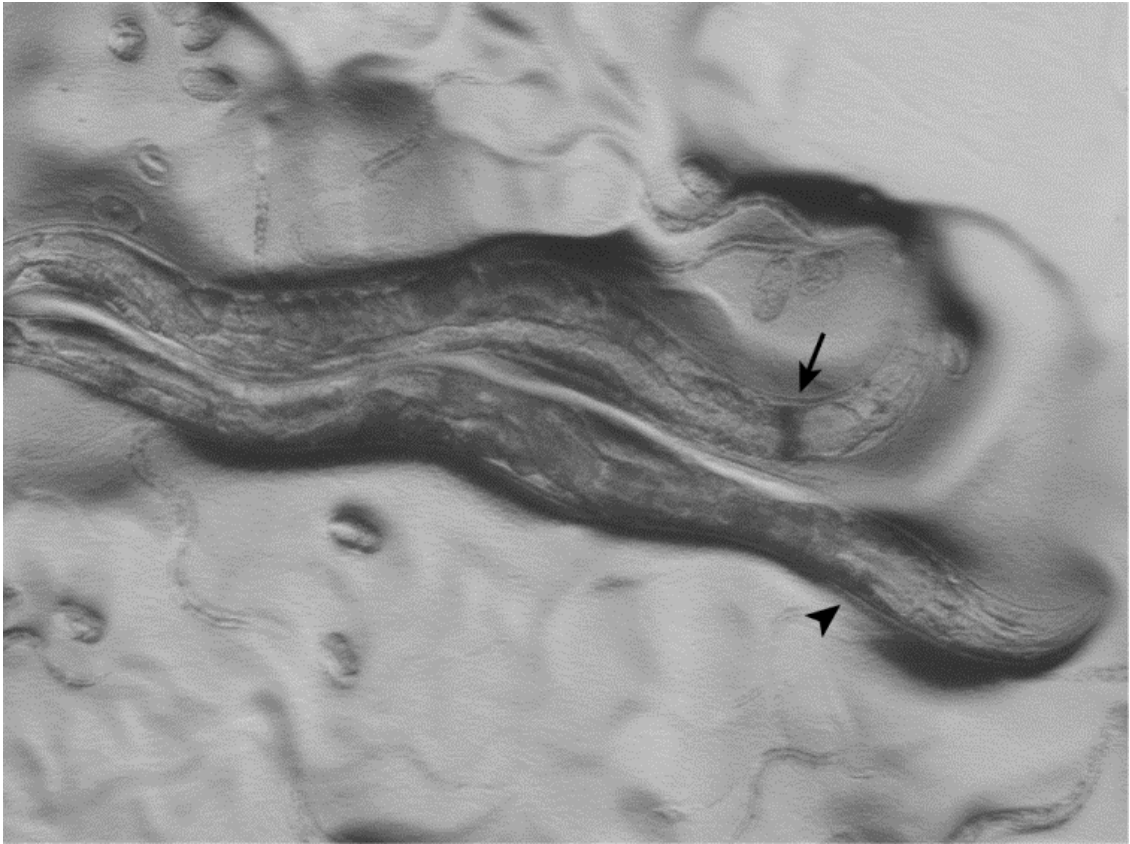


Figure S1: Ruby-throated phenotype. An individual with the ruby-throated phenotype (localized *Serratia marcescens* infection indicated by arrow) next to an uninfected individuals (lack of infection in the same region of the intestine noted by arrowhead). Photo credit: Erik Ragsdale with a Leica M205FA Stereo Microscope, Indiana University Light Microscopy Imaging Center.

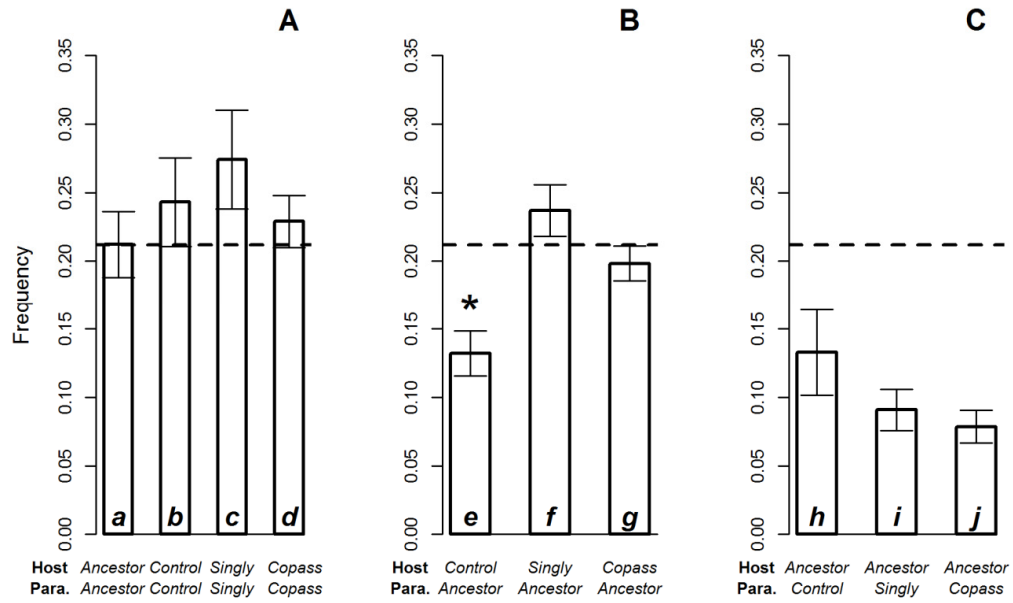


Figure S2: Frequency of the ruby-throated phenotype. The frequency of the ruby-throated phenotype across different pairings of host and parasite populations. (A) Host and parasite pairings according to shared evolution history. Frequency of the ruby-throated phenotype did not differ between pairings. (B) Hosts paired with ancestral parasites. The ruby-throated phenotype was significantly less frequent when control host lineages were paired with ancestral parasites. (C) Parasites paired with ancestral hosts. We did not observe any significant differences in the frequency of the ruby-throated phenotype between these pairings. The dashed line marks the starting point of experimental evolution: the mean frequency of the ruby-throated phenotype arising from pairings of ancestral hosts with ancestral parasites. Each bar is an average frequency obtained from 15 assay plates (3 assay plates per each of five lines), and error bars give the standard error of the mean frequency. Individual bars are referred to by letter in the text.

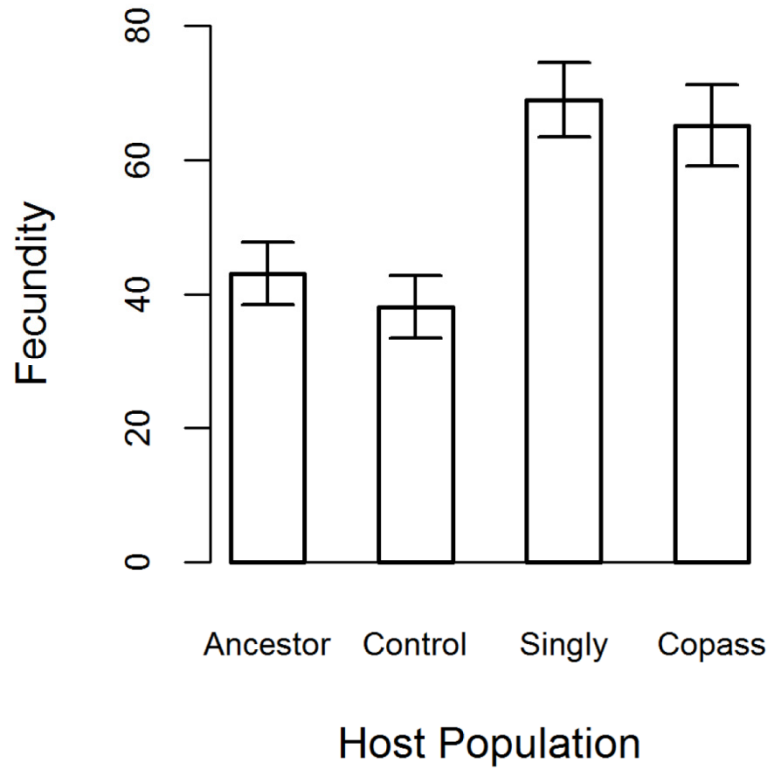


Figure S3: Fecundity of healthy hosts. The fecundity of uninfected hosts was measured approximately 48 hours into adulthood. Our experimental evolution design imposed selection for delayed reproduction, but we found no significant evidence that a shift towards delayed reproduction could explain the increased fecundity of ruby-throated hosts in the co-passaged treatment. Each bar is an average of the fecundity counts obtained from 75 hermaphrodites (15 hermaphrodites per each of five lines), and error bars give the standard error of the mean fecundity count.

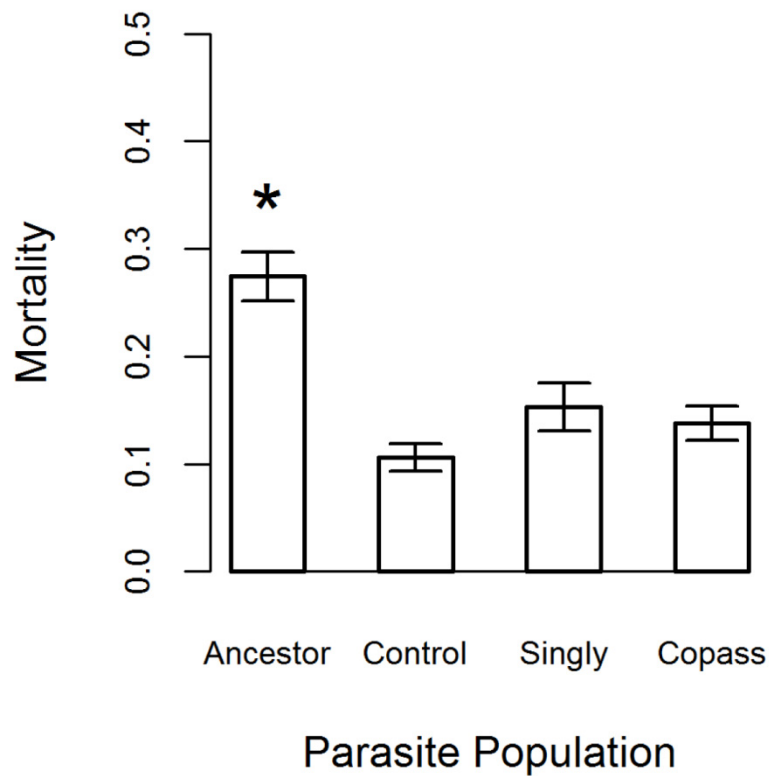
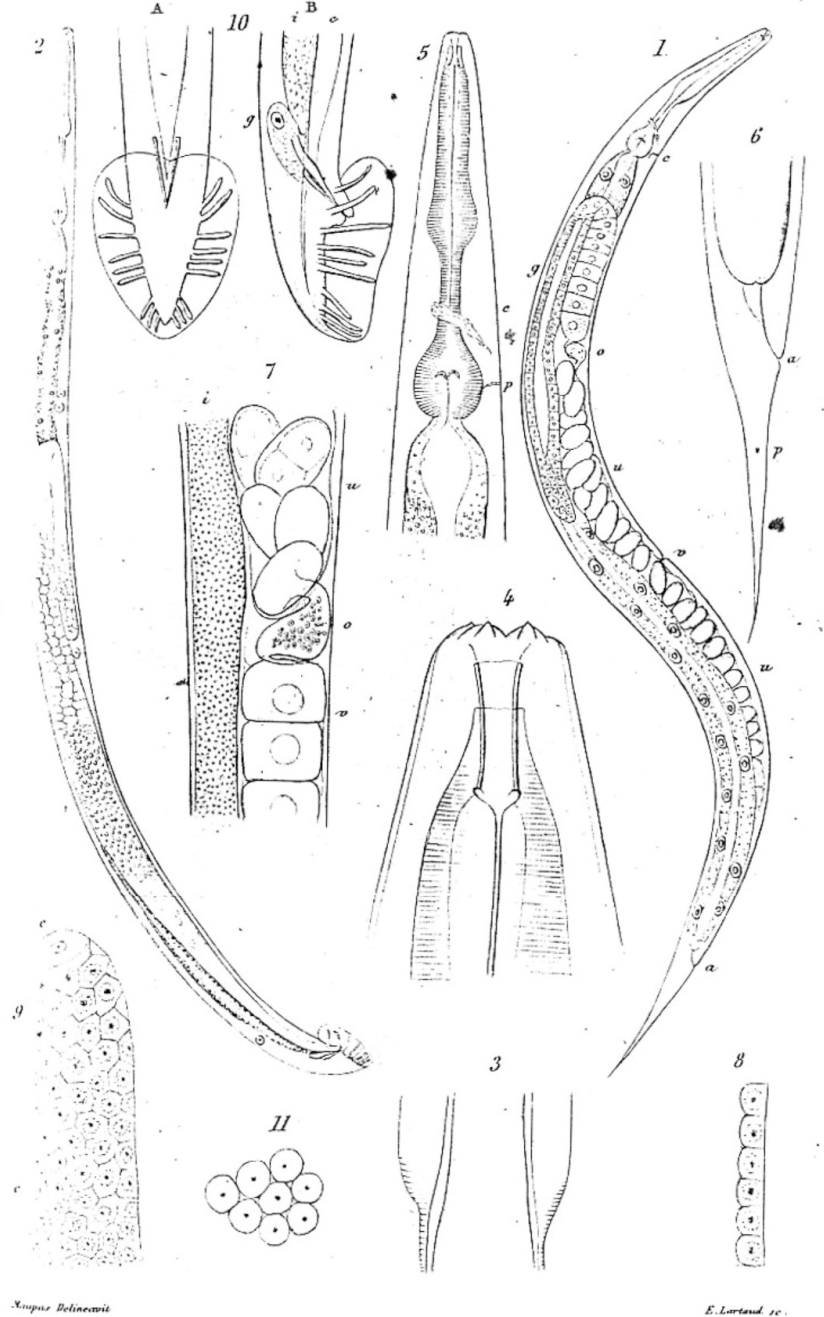


Figure S4: Virulence of parasite populations. Virulence of ancestral and experimental parasite populations was measured as the 24-hour mortality rate of each parasite population against ancestral hosts. The virulence of the ancestral parasite significantly exceeded that of control, singly-passaged, and co-passaged parasite populations, which did not significantly differ from one another. Each bar is an average of the 24-hour mortality rate measured for 20 populations (4 replicate assay plates per each of five lines), and error bars give the standard error of the mean mortality rate.

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Xenopus Delinensis

RHABDITIS ELEGANS

Librairie C. Reinwald

E. Lortaud sc.

Caenorhabditis elegans: from Maupas, E. 1990. Modes et formes de reproduction des nematodes. *Archives de Zoologie Expérimentale et Générale* 8: 463-624, the first description of *C. elegans* as a new species, originally as *Rhabditis elegans*. Image provided by scientificillustration.tumblr.com.

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Appendix I

The Red Queen's race: an experimental card game to teach
coevolution

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Abstract

Although we are increasingly aware that an understanding of evolution is critical to all biological fields and to scientific literacy, evolution remains a challenge in the classroom. Here we present a hands-on, inquiry-based classroom activity to study host-parasite coevolution. Coevolution is the reciprocal evolution of interacting species. It is pervasive, diverse, and rapid. Instruction in coevolution is therefore an excellent way to teach students evolutionary principles. In the described game, students take on the role of either host or parasite, and they use playing cards to act out reciprocal selection. Students collaborate to collect data on the change in frequency of host and parasite genotypes (card suits) through time. They use these data to conduct an independent test of the prediction that host-parasite coevolution maintains genetic variation. The game is suitable for students ranging from upper-level high school through college. We include detailed instructions, discussion topics, and simple modifications to extend the game to additional topics. This is a fun, active, and simple exercise to introduce students to the complex topic of host-parasite coevolution. Moreover, the game emphasizes infectious diseases as major selective forces, a fascinating topic for today's students.

Introduction

Since its origins in the latter half of the 19th century, the field of evolutionary biology has made enormous strides. We now recognize evolution as a unifying framework for the biological sciences. There are more and more calls for the incorporation of evolutionary principles into applied fields (Denison et al. 2003; Antonovics et al. 2007; Read and Huijben 2009). For example, the evolutionary medicine

movement seeks to incorporate evolutionary thinking into medical and pre-medical training (Nesse et al. 2010; Antolin et al. 2012). Education in evolutionary biology must grow to match our increasing awareness of the centrality of this field. Currently, the conceptual nature of evolution is notoriously difficult to teach, and the topic is fraught with misconceptions and complexity (Bishop and Anderson 1990; Alters and Nelson 2002; Dagher and Boujaoude 2005; Hokayem and BouJaoude 2008; Cunningham and Wescott 2009).

Thanukos (2010) recently made a compelling case that instruction on the topic of coevolution could tackle many of the conceptual hurdles that students face in understanding evolution. Coevolution is the reciprocal evolution of interacting species. It is pervasive, diverse, and very rapid. Instruction in coevolution can thus work to dispel the misconception that evolution acts only over long time-scales in response to abiotic changes, such as glaciation. Cases of coevolution demonstrate that evolution can occur over the course of only a few generations in response to continual change in the biotic environment. Moreover, coevolution powerfully contradicts the common notion of evolution as progressing towards a higher goal. In interactions between species, evolutionary optima are forever shifting in response to changes in the biotic environment (Jaenike 1978; Thompson 2005; Thanukos 2010).

In spite of this potential utility as a teaching tool in classrooms, Thompson (2010) notes that coverage of coevolution in biology textbooks is lacking. It is a challenging topic to teach via active laboratory exercises due to the complexity of balancing two or more interacting, adapting players. Here, we introduce a hands-on, inquiry-based activity to help students understand host-parasite coevolution. We developed it in order to

provide students with a test of the Red Queen Hypothesis for the maintenance of genetic variation, which we discuss in more detail in the next section. Students work in small groups using playing cards to generate the coevolutionary trajectories of hosts and parasites. They personally discover, through the fate of their own card hands, the rapid rate of reciprocal adaptation and the potential for host-parasite interactions to maintain diversity.

Host-parasite coevolution

The Red Queen Hypothesis (RQH) predicts that coevolution between hosts and parasites acts to maintain genetic variation through time. We developed this activity so that students could test this prediction and, in doing so, work through a classic model of host-parasite coevolution. The RQH posits that parasites adapt to specifically infect the most common host genotypes in a population. Parasites thereby exert negative frequency-dependent selection on their hosts, with the most common host genotypes having low fitness and declining in frequency as parasites infect them. Rare host genotype escape infection and increase in frequency (Haldane 1949; Jaenike 1978; Hamilton 1980; Hamilton et al. 1990). This rare advantage drives continual oscillations through time in the frequency of host genotypes and their matching parasite genotypes (Fig. 1A) (Hutson and Law 1981; Nee 1989). Thus coevolution is proposed to maintain genetic diversity in host and parasite populations (Jaenike 1978; Bell 1982).

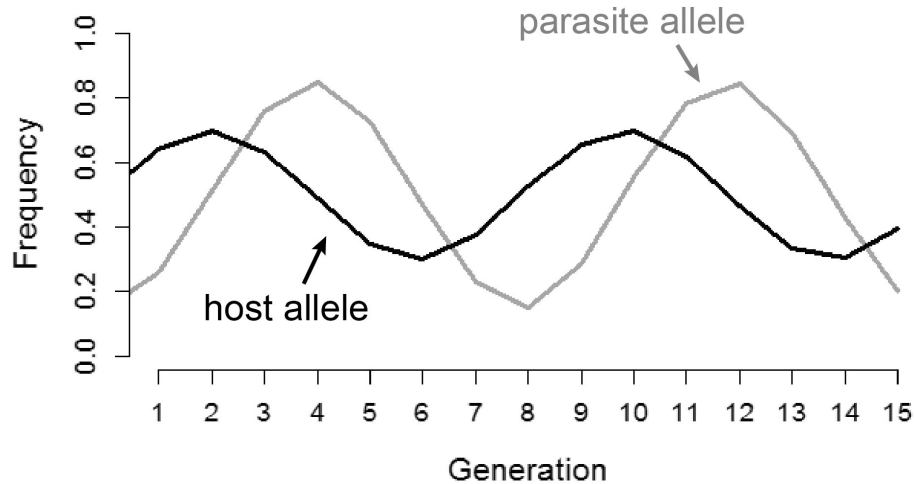
A key assumption of the RQH is that genetic matching is required for successful infection (Jaenike 1978). The most common model of infection genetics for studying Red Queen dynamics is the simple matching-alleles model (e.g. Hamilton et al. 1990; Howard

and Lively 1994). This model evokes the idea of self-non-self recognition: infection occurs when the host fails to recognize the parasite as a foreign entity. A host is susceptible to infection (i.e. the parasite is successful) if the host and parasite have matching genotypes. In contrast, a host is resistant to infection (i.e. the parasite is unsuccessful) if host and parasite genotypes are mismatched (Fig. 1B). Under this model, there is a strong trade-off to specialization, such that a parasite genotype is infective to only a fraction of host genotypes (Frank 1993).

The RQH itself is a powerful educational tool. First, host-parasite coevolution demonstrates to students that evolution can be a rapid, dynamic process. Secondly, the RQH focuses on parasites and infectious diseases, a timely and engaging topic. The topic particularly appeals to biomedical students, who make up a large fraction of biology majors. An understanding of infectious disease biology is of grave importance in today's world. The public are much more aware of disease outbreaks in human and non-human populations. The RQH can help students understand these events: it teaches the basics of infection genetics and makes predictions for the evolution of host and parasite populations over the course of epidemics. Similarly, the rise of antibiotic resistance is both a striking example of modern-day evolution in action and a pressing public health threat (Task Force for Combating Antibiotic-Resistance Bacteria 2015). The RQH can teach students some key evolutionary aspects of the problem: populations respond rapidly to strong selection, and parasites relentlessly adapt to host defenses. It is our moral obligation to train students on these issues, particularly students destined for medical professions (Antonovics et al. 2007; Read and Huijben 2009; Nesse et al. 2010; Antolin et al. 2012). We feel that teaching host-parasite coevolution in the context of the RQH is

Red Queen Dynamics

A



Matching-Alleles Model

B

		parasite allele			
		♣	♦	♥	♠
host allele	♣	+	-	-	-
	♦	-	+	-	-
	♥	-	-	+	-
	♠	-	-	-	+

Figure 1: Red Queen model of host-parasite coevolution. (A) Schematic of oscillations through time in the frequency of a host genotype (black) and its matching parasite genotype (gray). Host genotypes decline when common and increase when rare due to negative frequency-dependent selection exerted by the coevolving parasite population. The time-lag in the parasite genotype frequency reflects the period required for the parasite population to adapt to the changing host population. (B) Diagram of the matching-alleles model, as implemented in the game. Successful infection (+) results when the parasite genotype, represented here by a single allele or card suit, matches the host genotype. The host resists infection (-) when the host and parasite allele are mismatched.

an excellent way to accomplish this goal. We propose a classroom activity that is inexpensive, fun, and enlightening. The game provides an entry point for students to discover the basics of host-parasite coevolution.

The Red Queen's Game

Our game is effectively a simulation in which students take on the roles of host and parasite populations. This role-playing is the key to student engagement and comprehension. Students playing the host not only see the oscillations in genotype frequency in the data they generate: they personally dread the adaptation of the parasite population to their host hand and suffer the inevitable crash of the host population. Likewise, students playing the parasite take satisfaction from the growing success of the parasite population as it adapts to the host population and lament its failure as the composition of the host hand rapidly shifts away in response. We present the game to the students as a means to test the key prediction of the RQH: host-parasite coevolution maintains genetic variation. The students use the game to see for themselves if this prediction is upheld. In other words, the students are engaged in hypothesis testing.

Our goal for this exercise is to convey four general concepts (Fig. 2A): 1. Coevolution occurs rapidly; 2. That which is most fit now can become the least fit in just a few generations; 3. Rare advantage, or negative frequency-dependent selection, can maintain genetic variation over time; and 4. We can use simple games to represent complex processes and to test hypotheses. Prior to beginning the game, we encourage instructors to pose four questions to their students that will emphasize these concepts. Presented in Figure 2B, these “warm-up” questions ask students to reflect upon their

initial understanding of coevolution and how we might study it. They will struggle to answer these questions prior to playing the game. By guessing and discussing answers with their peers, however, students will be thinking about the key concepts of the game as they begin playing.

Materials

The only required materials for the game are two decks of playing cards per group of students, one deck for the host population and one for the parasite population. It is best to use two distinct decks to facilitate separation of the host and parasite populations (e.g. blue-backed decks for host, red-backed for parasite). We have made additional resources available online at <http://www.indiana.edu/~curtweb/EvolutionLabs/> and in Additional files 1 and 2 (a worksheet with directions and a spreadsheet for data entry and calculations). These include a spreadsheet for data entry and calculations, directions, and a worksheet. We provide this spreadsheet to students as a Google sheet and give all students access and editing privileges. Students enter their data directly into the Google sheet, and we project the results of the game live. This set-up facilitates sharing of the data between all groups. It requires that each group of students have access to a computer for data entry.

Game set-up

We present brief introductory material to the students and then instruct them to split into groups, collect card decks, and read the directions thoroughly. We conduct this exercise with students in groups of two: one student playing the host population and one playing

A. Concepts

1. Coevolution is rapid
2. That which is most fit now can soon become the least fit
3. Rare advantage maintains genetic variation over time
4. Simple games can capture complex processes and test hypotheses

B. Warm-up questions

1. How fast is coevolution? How many generations (i.e. rounds of play) do you expect it to take to see evolutionary change in the host or parasite population?
2. How would you find the most fit genotype in a population? How would you measure change in fitness over time?
3. Do you think natural selection favoring rare genotypes will maintain genetic variation in a population? Why? How would you test this?
4. What factors impact species interactions in nature? How?

C. Wrap-up questions

1. Have the results of the game changed your views on the rate of coevolution? Why?
 - Compare the oscillations of your host and parasite genotype frequencies. For a given suit, are the oscillations of host and parasite perfectly in sync? If not, is host or parasite lagging, and by how many generations? Why?
2. Now, with data in hand, how would you propose we assess whether a genotype is the most fit in a population? And how would you measure changes in fitness over time?
 - Calculate the change in frequency of each host genotype from generations 1 to 4. Which genotype increases the most?
 - Calculate the change in frequency of each host genotype from generations 4 to 8. Which genotype increases the most? Is it the same genotype as the previous answer?
3. Did natural selection favor rare genotypes in this game? Why or why not? How would you propose we assess whether or not genetic variation was maintained?
 - Apply your suggestion – was genetic variation maintained? Consider genetic variation in both the host and the parasite population in your answer.
 - What do you predict would happen to host genotype frequencies over time if parasites were absent? Sketch your prediction. Design a modification of the game to test your prediction.
4. Do you think all coevolution can be described by these “Red Queen dynamics?” What’s an alternative?

D. Metapopulation thinking

- Why do the individual populations differ so dramatically from one another? Why does the metapopulation differ so dramatically from the individual populations?
- Based upon the metapopulation data, is any one genotype inherently most fit?
- Imagine that we allowed migration between populations: on average, would a migrant host’s fitness be greater or less than the local hosts? A migrant parasite’s?

Figure 2: Concepts and questions for classroom discussion. We outline (A) four central concepts of the game, (B) warm-up questions to emphasize these concepts, (C) wrap-up questions in which students revisit and revise their responses to the warm-up questions, and (D) questions for discussing the metapopulation level of the game.

the parasite population. Students are advised to switch halfway through so that they can personally experience both roles. We suggest students play for 15 generations, which requires a minimum of 45 minutes.

The sequence of the game is simple, and students catch on after 1-2 generations of independent play. The game is amenable to modification according to the level and size of the class and to the desired learning goals. Here, we outline the specific approach that we have used for playing the game with 24 undergraduate students in CM Lively's Evolution course at Indiana University.

Students begin by establishing their starting host and parasite populations. They shuffle their respective decks and randomly select 12 cards. The remaining cards become the reserve deck. Each suit is a genotype – clubs, spades, hearts, and diamonds. Students count the number of individuals of each “genotype” in their hand and record these data in the generation 0 row of their data sheet (spreadsheet provided online and in Additional file 2). Students should ensure that these counts sum to 12 for both the host and parasite populations: the provided spreadsheet includes a column for this purpose. We additionally constructed the spreadsheet such that counts are automatically translated into frequencies and plotted. For example, if half of the cards held by the “host” are spades, then the frequency of the spade genotype is 0.5.

Four basic steps constitute a single “generation” of this game (Fig. 3):

Step 1: host-parasite contact.

Host and parasite shuffle their populations. They then work together to randomly pair each host and parasite card, resulting in 12 host-parasite pairs. Students tend to find

their own way of efficiently performing this step. For example, the host student could lay out her cards, and then the parasite student can lay out his cards next to the host cards until 12 pairs are formed. This step requires that each group have enough work space to lay out their pairs. It is also helpful for host and parasite decks to be distinct in some way (e.g. different backs, say red and blue), so that host and parasite individuals can be separated following the selection step.

Step 2: infection and selection

According to the matching-alleles model for infection genetics, a parasite successfully infects a host when its genotype matches that of its host (Frank 1993). Therefore, infection results for those pairs in which the host and parasite genotype match (e.g. host and parasite are both spades) (Fig. 1B). The host individual is sterilized or killed, so the host student discards that card by placing it in her reserve deck. The parasite student retains the successful parasite card for subsequent reproduction. For pairs in which the host and parasite genotype do not match, the host resists infection. The parasite does not survive the failed infection (as in Salathé et al. 2008; King et al. 2011b), so the parasite student discards it by placing it in his reserve deck. The host student retains the successful host card for reproduction. In this formulation of the game, each parasite individual has only this single chance to infect. Students will often find that matches (successful infections) are rare in the initial generations of the game and increase through time as the parasite population adapts.

Step 3: reproduction

Each surviving host makes two offspring and dies. Students simulate this process by adding one card of the matching suit for each surviving host card (for a total of two cards of the same suit). Each successful parasite makes three offspring and dies. Students simulate this process by adding two cards of the matching suit for each surviving parasite card (for a total of three cards). Sometimes, a student's reserve deck does not have enough cards of a given suit to give each surviving individual enough offspring. In this case, the student should randomly select cards from the reserve deck until all individuals have reproduced. These randomly selected offspring will not match the genotype of the parent; students can think of this step as mutation.

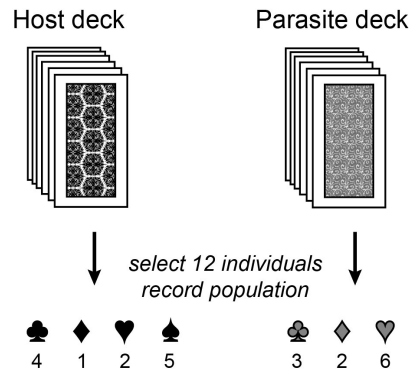
We feel that the greater offspring number of parasites relative to hosts (3 v. 2) is biologically realistic. Computer simulations also demonstrated that this tends to generate smoother oscillatory dynamics than equivalent offspring numbers: it increases the probability of matching by facilitating rapid evolution of the parasite (data not shown).

Step 4: population size regulation

Students rarely have exactly 12 individuals at the end of the reproduction step. The population nonetheless remains fixed at 12. If populations have too few offspring (common for the parasite population in particular), students should randomly select offspring from the reserve deck until they have 12 offspring. This step can be thought of as immigration. If populations have too many offspring (common for the host population), students should shuffle the offspring and randomly select 12 cards to make the next generation. They should return the remainder to their reserve deck. This step is

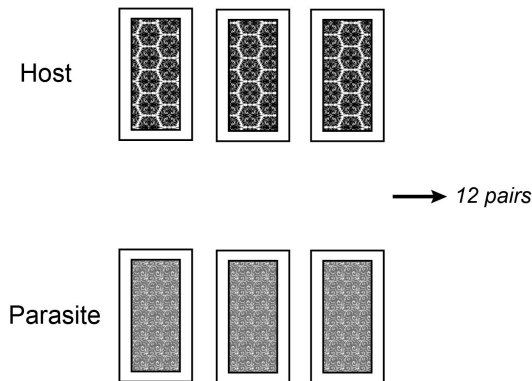
SET-UP

Establish starting populations



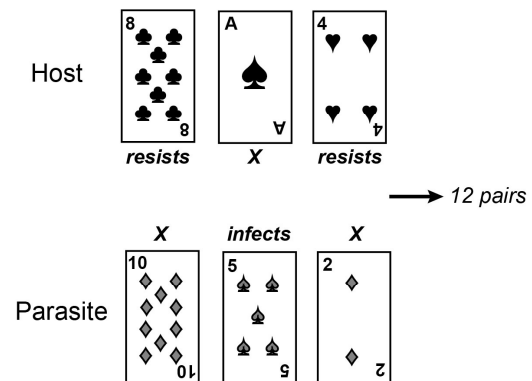
STEP 1

Host-parasite contact



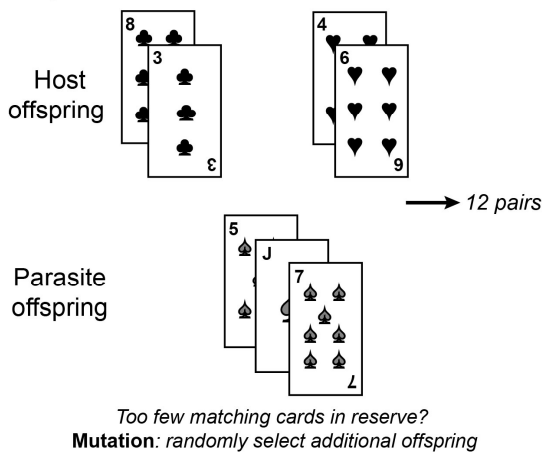
STEP 2

Infection and selection



STEP 3

Reproduction



STEP 4

Population size regulation

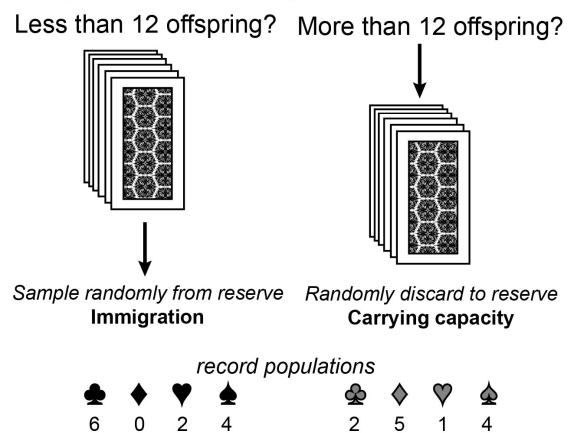


Figure 3: Schematic of the set-up and four basic steps of the Red Queen game.

These four steps constitute a single generation of play. We propose 15 generations of play (i.e. 15 repetitions of these steps) for a classroom exercise.

consistent with a carrying capacity for the population. The students then record the number of individuals of each genotype under generation 1 of the spreadsheet. Repeat steps 1-4 for 14 more generations. We find that 15 generations is sufficient to obtain 3-4 oscillations (Fig. 4).

Outcome

After 15 generations, the students will have generated host and parasite genotype frequencies through time. If using a data entry system that allows live updates and data sharing, each group will also have access to the data and plots of other groups in the class. In Figure 4, we show sample data generated by our own students (raw data in Additional file 3). Oscillations in host and parasite genotype frequencies, with a time-lag of a few generations, are obvious (Fig. 4A-D). The specific follow-up exercises that an instructor wishes to follow should be tailored to the level of the students, the prior coverage of these topics in the class, and the instructor's specific educational goals (see sample handout in supplemental material). We propose several questions to return students to the game's key concepts. Presented in Figure 2C, these "wrap-up" questions ask students to revisit, and perhaps revise, their answers to the warm-up questions (Fig. 2B). In answering them, students use the data they've generated to measure time lags in parasite adaptation, changes in host fitness over time, and genetic variation in the host and parasite populations.

We also encourage instructors to show students the results at the level of the "metapopulation," meaning across all populations. We propose this for several reasons. First, coevolution leads to divergence between populations: as a host and parasite

population reciprocally adapt, they can adopt distinct evolutionary trajectories from their neighbors, just by chance alone. Students will see this when they compare allele frequencies at generation 15 in different groups (Fig. 4A-D). Secondly, no genotype has an inherent fitness advantage in the game: fitness is determined solely by the frequency of a genotype's matching partner. This is an unusual idea that is obvious in the metapopulation data: each host and parasite genotype is maintained at ~25% of the metapopulation, and the oscillations are damped (Fig. 4F). Finally, we have come to realize that coevolution must be considered at the metapopulation level (Thompson 2005): for example, moderate gene flow between populations can promote coevolution by increasing genetic variation (Gandon et al. 1996; Lively 1999; Gandon and Michalakis 2002; Greischar and Koskella 2007). This exercise exposes students to this kind of metapopulation thinking. The provided spreadsheet for data entry includes a tab to calculate and plot the average host and parasite genotype frequencies across all groups of students (Fig. 4). In Figure 2D, we present three discussion questions that highlight these key metapopulation points: the striking divergence between populations, equal mean fitness of all genotypes, and migration between populations.

Extensions

Discussion topics

We recommend several published articles for discussion that would pair nicely with the above activity. For example, Chaboudez and Burdon (1995), Lively et al (1990), and Wolinska and Spaak (2009) provide simple and compelling studies of frequency-dependent selection by parasites in natural systems. Chaboudez and Burdon (1995)

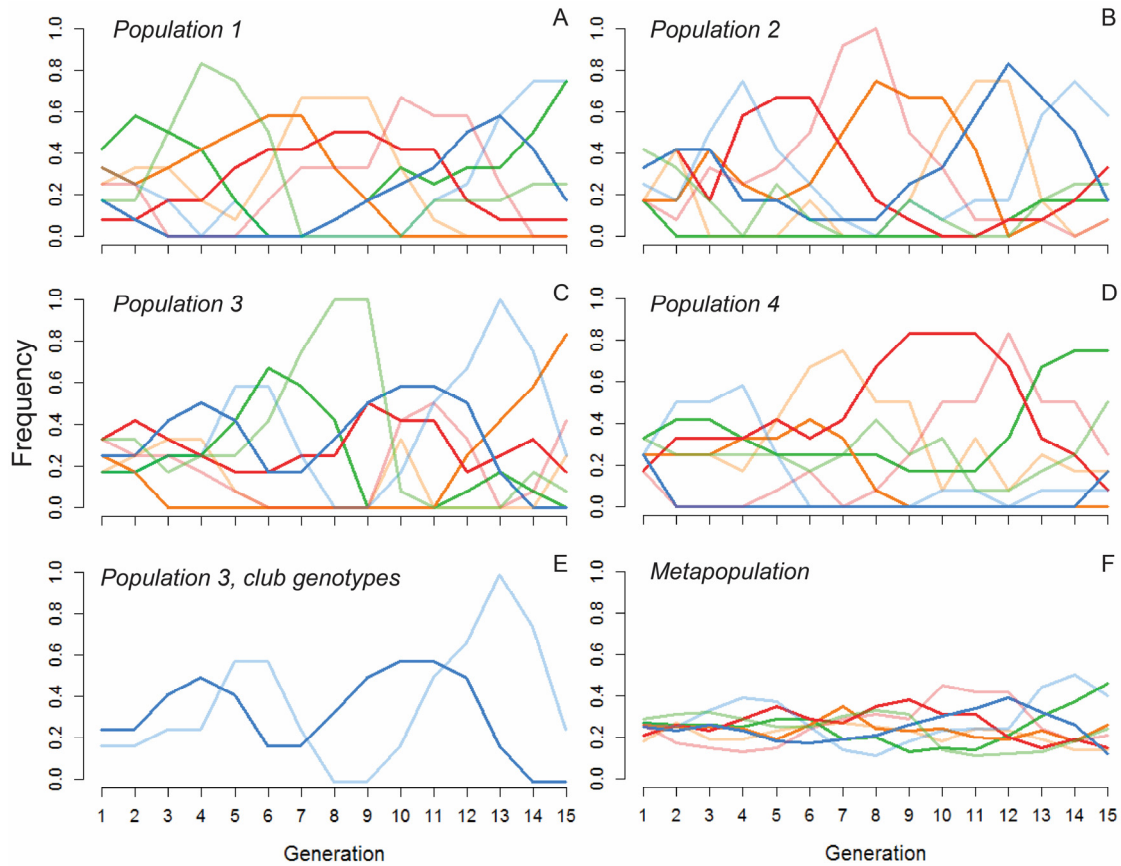


Figure 4: Red Queen dynamics in sample game data. Seven groups of students each played the Red Queen game for 15 generations during a class period of Indiana University’s S318 Honors Evolution course. (A-D) show the oscillations in frequencies of host (bold lines) and parasite (faded lines) genotypes (suits) over 15 generations for four different populations (student groups). Frequencies are derived from the numbers of host and parasite individuals of each genotype recorded at each generation (step 4). Matching host and parasite genotype frequencies are displayed in the same color (e.g. the club genotype is in bold blue for host and faded blue for the parasite). Each group experienced multiple oscillations in genotype frequencies, and the trajectories differ widely between groups. (E) highlights the time-lagged nature of oscillations for a single matching pair of host (bold) and parasite (faded) genotypes (clubs). Data derived from population 3 above. (F) shows the dynamics of the metapopulation: the average frequency of each host and parasite genotype across seven groups (the four shown above plus three additional). The oscillations in genotype frequencies are damped, which we would predict based upon the fact that the game does not include any inherent fitness differences between genotypes. Raw data in Additional file 3.

surveyed natural populations of a flowering plant infected with a rust fungus to test the Red Queen's prediction that parasites adapt to infect the most common host clones in a population. Their data support this prediction: the most common clone was the only infected clone in the majority of host populations, a greater fraction than expected by random chance. Students could apply the Chaboudez and Burdon (1995) approach to their simulated data: at generation 15, in what proportion of populations is the most common host genotype matched by the most common parasite genotype?

We developed this activity in conjunction with a unit on the evolution of sexual reproduction. The RQH arose to address the paradox of sexual reproduction (Jaenike 1978; Bell 1982). Asexual reproduction is far more efficient than sexual reproduction, so asexual lineages should outcompete sexual ones (Maynard Smith 1978; Lively and Lloyd 1990; Lively 1996). Why then is sexual reproduction so common? The Red Queen argues that host-parasite coevolution favors sexual individuals because they produce offspring with a variety of rare genotypes. In contrast, parasites rapidly adapt to infect common asexual lineages and drive them down in frequency (Haldane 1949; Jaenike 1978; Hamilton 1980; Hamilton et al. 1990). To further address hypotheses for the maintenance of sex and genetic variation, we recommend Burt and Bell (1987). Their study provides an excellent example of the strong inference approach to hypothesis testing. It also encourages students to reflect upon the evolutionary conditions under which we predict selection for elevated recombination. Lastly, Hamilton and Zuk (1982) is a classic work that would serve to connect host-parasite coevolution to a unit on sexual selection.

Variations on the game

An exciting aspect of the game we outline here is that the basic framework can be modified in small ways to test additional predictions. Instructors can ask students to design their own modifications to test new predictions they have made based upon class data (e.g. Fig. 2C, question 3). Here, we highlight four of the many modifications that might be interesting to pursue in the classroom.

First, to drive home the idea that coevolution acts to maintain genotypic diversity, the students can contrast the original game with one in which selection is absent. In this case, students play the game as before, but there are no fitness consequences of successful or unsuccessful infection for either host or parasite. In other words, parasite virulence is zero, and there is no cost for a parasite of a failed infection. Students should predict in advance that genetic variation will not be maintained under these conditions, because rare advantage is absent.

Secondly, students can further test the advantage of a rare host genotype by beginning the game with no genetic variation. In this modification, the game starts with a single suit and variation is introduced through mutation (in step 3) and migration (in step 4). Students should predict in advance that mutant or migrant host genotypes will rapidly increase in frequency. In contrast, mutant or migrant parasite genotypes will be less successful, as they are unlikely to match the most common host genotype.

This suggests a third modification: for advanced classes, instructors might consider introducing the concept of local adaptation using data generated by the game. Coevolution is predicted to result in local adaptation, in which parasites have higher fitness when infecting their sympatric host population than allopatric host populations

(Parker 1985; Lively 1989). By calculating mean fitness of each parasite population on the host population of each group at generation 15, we indeed find local adaptation of parasite populations (data not shown).

A final option is to explicitly test the Red Queen's prediction for the maintenance of sexual reproduction: coevolving parasites maintain sex in their hosts. In the game described here, all host individuals are clonal. Step 3 (reproduction) might be modified to compete sexual individuals against clones: a fraction of host individuals could be clonal, with the rest producing offspring in a manner that models recombination. There are of course many modifications besides these few that we highlight here: the game could be altered to use diploid rather than haploid individuals or to test the results obtained under different virulence levels. The option also exists to use custom card decks, rather than traditional playing cards, to have greater flexibility in the number of genotypes and individuals per genotype.

Conclusion

Pedagogical studies in biology and general science emphasize the efficacy of hands-on, inquiry-based activities that actively engage students in the learning process (Hake 1998; Alters and Nelson 2002; Smith et al. 2005; Nelson 2008). We have described a game in which students work cooperatively in small groups to generate their own data for an independent test of the central prediction of the Red Queen Hypothesis. In pursuing this specific goal, students personally engage with the broader concepts of rapid coevolution and frequency-dependent selection. We offer this basic exercise as a

fun and inexpensive tool for teaching evolution at the undergraduate and advanced high school level.

Acknowledgements

We first and foremost thank the students of Indiana University's Honors Evolution S318 course in the fall of 2014 for inspiring this activity, test driving it, and giving helpful feedback. We are grateful to Amrita Bhattacharya for helping test early versions of the game, as well as Amy Dapper, Marta Shocket, and Mikus Abolins-Abols for their valuable input. AK Gibson recognizes funding from the National Science Foundation's Graduate Research Fellowship Program. We thank Albert and Kathy Ruesink for establishing the Albert Ruesink Outstanding Associate Instructor Teaching Award in Biology. This award to AKG contributed to publication costs.

Amanda Kyle Gibson, CV

Education

2010 – 2016 PhD Evolution, Ecology and Behavior, Indiana University; Advisor: C Lively
2004 – 2008 B.A. Biology, *summa cum laude*, Amherst College; Advisor: M Hood

Research Appointments

2009 – 2010 Intramural Research Trainee: National Institutes of Health; Advisor: M Grigg
2008 – 2009 Fulbright Scholar: l'Université de Paris-Sud XI, France; Advisor: Tatiana Giraud
Summer 2006 Research Intern: National Institutes of Health; Advisors: A Báfica, A Rothfuchs

Teaching Appointments

2015 Associate Instructor: L111 Evolution and Diversity, Indiana University
2014 Associate Instructor (volunteer): S318 Honors Evolution, Indiana University
2010 Associate Instructor: L111 Evolution and Diversity, Indiana University
2007-2008 Teaching Assistant: BIO 18 Adaptation and the Organism, Amherst College

Publications (*undergraduate mentees bolded*)

18. AK Gibson, **JY Xu**, and CM Lively. Within-population covariation between sexual reproduction and susceptibility to local parasites. *Evolution*, in press.
17. AK Gibson, J Jokela, and CM Lively. 2016. Fine-scale spatial covariation between infection prevalence and susceptibility in a natural population. *The American Naturalist* 188(1): 1-14.
16. MJ McKone, AK Gibson, ...M Neiman. 2016. Fine-scale association between parasites and sex in *Potamopyrgus antipodarum* within a New Zealand lake. *New Zealand Journal of Ecology* 40(3).
15. DM Castillo, AK Gibson, and LC Moyle. 2016. Assortative mating and self-fertilization differ in their contributions to reinforcement, cascade speciation, and diversification. *Current Zoology* 62(2): 169-181.
14. AK Gibson, **KS Stoy**, **IA Gelarden**, **MJ Penley**, CM Lively, and LT Morran. 2015. The evolution of reduced antagonism - a role for host-parasite coevolution. *Evolution* 69(11): 2820-2830.
13. AK Gibson, DM Drown and CM Lively. 2015. The Red Queen's Race: an experimental card game to teach coevolution. *Evolution: Education and Outreach* 8: 10. *Huxley Award for evolution education*
12. AK Gibson and JA Fuentes. 2015. A phylogenetic test of the Red Queen Hypothesis: outcrossing and parasitism in the Nematode phylum. *Evolution* 69(2): 530-540.
11. L Barbosa, CK Johnson, DM Lambourn, AK Gibson, ...ME Grigg. 2015. A novel *Sarcocystis neurona* genotype XIII is associated with severe encephalitis in an unexpectedly broad range of marine mammals from the northeastern Pacific Ocean. *International Journal for Parasitology* 45(9-10): 595-603.
10. AK Gibson, G Refrégier, ME Hood, and T. Giraud. 2014. Performance of a hybrid fungal pathogen on pure species and hybrid plant hosts. *International Journal of Plant Sciences* 175(6): 724-730.

9. AK Gibson, Z Smith, C Fuqua, K Clay, and JK Colbourne. 2013 Why so many unknown genes? Partitioning orphans from a representative transcriptome of the lone star tick *Amblyomma americanum*. *BMC Genomics* 15: 135.
8. AK Gibson, E Petit, J Mena-Ali, B Oxelman, and ME Hood. 2013. Life-history strategy defends against disease and may select against physiological resistance. *Ecology and Evolution* 3: 1741-1750.
7. AG Rothfuchs, E Roffe, AK Gibson, ... A Bafica. 2012. Mannose-binding lectin regulates host resistance and pathology during experimental infection with *Trypanosoma cruzi*. *PLoS One* 7(11): e47835.
6. AK Gibson, ME Hood, T Giraud. 2012. Sibling competition arena: selfing and a competition arena can combine to constitute a barrier to gene flow in sympatry. *Evolution* 66: 1917-1930.
5. JM Wendte, AK Gibson, ME Grigg. 2011. Population genetics of *Toxoplasma gondii*: new perspectives from parasite genotypes in wildlife. *Veterinary Parasitology* 182: 96-111.
4. AK Gibson, S Raverty, DM. Lambourn,...ME Grigg. 2011. Polyparasitism is associated with increased disease severity in *Toxoplasma gondii*-infected marine sentinel species. *PLoS Neglected Tropical Diseases* 5(5): e114.
3. M López-Villavicencio, F Courjol, AK Gibson,...T Giraud. 2011. Competition, cooperation among kin, and virulence in multiple infections. *Evolution* 65: 1357-1366.
2. AK Gibson, J Mena-Ali, ME Hood. 2010. Loss of pathogens in threatened plant species. *Oikos* 119: 1919-1928.
1. ME Hood, J Mena-Ali, AK Gibson, ...J Antonovics. 2010. Distribution of the anther-smut pathogen *Microbotryum* on species of the Caryophyllaceae. *New Phytologist* 187: 217-229.

AK Gibson, LF Delph, and CM Lively. The two-fold cost of sex: experimental evidence from a natural system. Submitted.

Research Grants

2016	Louise Constable Hoover Fellowship, IU	\$1000
2015	Common Themes in Reproductive Diversity, IU	\$900
2015	College of Arts and Sciences Graduate Student Travel Award, IU	\$500
2015	Provost's Travel Award, IU	\$1,700
2014	Senior Research Grant, Indiana Academy of Sciences	\$1,431
2014	Doctoral Dissertation Improvement Award, NSF	\$13,000
2013	Travel Award, Graduate and Professional Student Organization, IU	\$500
2013	Student Research Award, American Society of Naturalists	\$2,000
2012	Rosemary Grant Award, Society for the Study of Evolution	\$2,000
2012	Common Themes in Reproductive Diversity, IU and NIH	\$2,000
2011	Women in Science Travel Grant, IU	\$400
2011	Grant-in-Aid of Research, Sigma Xi	\$400

Fellowships and Awards

2016	Thomas Henry Huxley Award for education, Society for the Study of Evolution
2015 – 2016	Common Themes in Reproductive Diversity Traineeship, IU
2015	Ruesink Outstanding Associate Instructor Teaching Award in Biology

2010 – 2015	Graduate Research Fellowship, National Science Foundation
2012 – 2013	Common Themes in Reproductive Diversity Traineeship, IU and NIH
2013	Ruth Patrick Award for best poster at Evolution, American Society of Naturalists
2013	Lloyd I. Rosenblum Memorial Fellowship for graduate studies, Amherst College
2012	John Woodruff Simpson Fellowship for graduate studies, Amherst College
2011	Memorial Fellowship for graduate studies, Amherst College
2008-2009	Fulbright Fellowship for research at l'Université de Paris-Sud XI, France
2009	Young Investigator Award for best talk; FEBS Course, Cell Host and Microbe
2009	Young Investigator Award for best talk; FEBS Course, Eukaryotic Cell
2008	Oscar E. Schotté Scholarship to support summer research, Amherst College
2008	Oscar E. Schotté Award for the best senior thesis in Biology, Amherst College
2008	Phi Beta Kappa, Amherst College

Presentations

Invited presentations

2016	The two-fold cost of sex: experimental evidence from a natural system. WD Hamilton Symposium, Evolution . Austin, TX 18 June. <i>Honorable Mention, WD Hamilton Award</i>
2014	A phylogenetic test of the Red Queen Hypothesis: outcrossing and parasitism in the Nematode phylum. Departmental Seminar, Amherst College . Amherst, MA. 21 April
2013	A phylogenetic test of the Red Queen Hypothesis. 2nd International Microbotryum Meeting . Amherst, MA. 4 May
2010	Emergence of terrestrial pathogens in marine mammals. National Marine Animal Health and Stranding Network Conference . Shepherdstown, WV. 7 April

Contributed presentations

2015	Parasite-mediated selection on sex: what are its fitness consequences in nature? Congress of the European Society for Evolutionary Biology . Selected presentation. Lausanne, Switzerland. 10 August Fine-scale spatial variation in disease and susceptibility. Indiana Academy of Science Annual Meeting . Indianapolis, IN. 21 March
2014	Experimental evolution of reduced antagonism: a role for host-parasite coevolution. Evolution . Raleigh, NC. 21 June
2012	Selfing and the sibling competition arena. 1st Joint Congress on Evolutionary Biology . Ottawa, Canada. 9 July
2011	Loss of pathogens in threatened plant species. Conférences Jacques-Monod: New and Emerging Fungal Diseases of Plant and Animals . Selected presentation. Roscoff, France. 27 June
2009	Hybridization and phylogenetics in predicting pathogen emergence on a novel host. Third FEBS Advanced Lecture Course: Human Fungal Pathogens . Selected presentation. Nice, France. 3 May; <i>Cell Host and Microbe Young Investigator Award</i> ; <i>Eukaryotic Cell Young Investigator Award</i> Loss of pathogens and disease resistance in threatened plant species. Annual meeting of Le Réseau Ecologie des Interactions Durables . Amiens, France. 15 January

Posters

- 2015 Do coevolving parasites maintain genetic variation? A tale of spatial variation in infection, susceptibility, and sex. **Ecology and Evolution of Infectious Disease**. Athens, Georgia. 26 May
- 2013 A phylogenetic test of the Red Queen: outcrossing and parasitism in the Nematode phylum. **Evolution**. Snowbird, Utah. 23 May; *Ruth Patrick Student Poster Award*
The evolution of sex and parasitism in the phylum Nematoda. **Ecology and Evolution of Infectious Disease**. State College, PA. 21 May
- 2011 Coinfection is associated with increased disease severity in protozoa-infected marine mammals. **Early Career Scientists Symposium: Infectious Disease across Scales**. Ann Arbor, MI. 16 April
- 2009 Hybridization and phylogenetics predict pathogen emergence on novel hosts. Congress of the **European Society of Evolutionary Biology**. Turin, Italy. 23 August.
Hybridization and phylogenetics in predicting pathogen emergence on a novel host.
Third FEBS Advanced Lecture Course: Human Fungal Pathogens. Nice, France. 3 May

Undergraduate Mentoring

- 2013 – 2016 Sam Klosak, L490 Independent Research and flow cytometry technician, IU
- 2014 – 2015 Julie Xu, IFLE and STARS programs and L490 Independent Research, IU
- 2013 – 2015 Peyton Joachim, laboratory volunteer and L490 Independent Research, IU; now works in two biology labs and is preparing for his Senior Honors Thesis
- 2013 – 2014 David Nugent, laboratory assistant, IU; now completing his degree in Healthcare Management and Policy at IU
- 2012 – 2014 Kayla Mitman-Stoy, Senior Honors Thesis student, IU; now works in stem cell research at Cook Pharmica; **recipient of Outstanding Honors Thesis Award*
- 2013 – 2014 Ian Gelarden, Senior Honors Thesis student; now at IU School of Medicine
- 2013 Laura Sloan, L490 Independent Research, IU; now at Tufts Cummings School of Veterinary Medicine
- 2013 Justin Wyss, L490 Independent Research, IU; now at IU McKinney School of Law
- 2010 McKenna Penley, laboratory assistant, IU; now lab manager at Emory University

Undergraduate publications (undergraduate mentees bolded)

1. **PJ Joachim**, AK Gibson, and CM Lively. 2015. Research snapshot: evolution of resistance in *Potamopyrgus antipodarum*. *Indiana University Journal of Undergraduate Research* 1: 35.
2. **SA Klosak**, AK Gibson, LF Delph, and CM Lively. 2015. Research snapshot: parasite selection and the fitness of sexual reproduction. *Indiana University Journal of Undergraduate Research* 1: 36.
3. **JY Xu**, AK Gibson, and CM Lively. 2015. Research snapshot: the effect of host's dispersal ability on fine-scale spatial differentiation. *Indiana University Journal of Undergraduate Research* 1: 38.

Undergraduate presentations

- 2015 P Joachim. The evolution of resistance in *Potamopyrgus antipodarum*. **Midwest Ecology and Evolution Conference**. Bloomington, IN. 28 March. *Poster*
- KS Mitman-Stoy. Experimental evolution of reduced antagonism – a role for host-parasite coevolution. **Midwest Ecology and Evolution Conference**. Bloomington, IN. 28 March. *Talk*
- J Xu. Fine-scale spatial variation in coevolution and sexual reproduction. **Midwest Ecology and Evolution Conference**. Bloomington, IN. 28 March. *Poster*

Professional Activities and Service

Volunteer teaching

- 2016 Classroom volunteer for M375 Human Parasitology, IU. Implemented the Red Queen Game
- 2015 Volunteer lecture: “Evolution of drug resistance”; L111, IU
- 2015 Classroom volunteer for Advanced Placement Biology, Bloomington High School South, IN; Implemented the Red Queen Game
- 2014 + 2015 Evolution Teacher, Jim Holland Summer Enrichment Program for underrepresented students, IU
- 2014 Classroom volunteer with IU Biology Outreach, Templeton Elementary, IN
- 2014 Volunteer Assistant Instructor for S318 Honors Evolution, IU; specific activities included tutoring in science writing, development of the Red Queen Game, lecturing on phylogenetics, and development of phylogenetics laboratory exercises ; *work recognized by Ruesink Outstanding Teaching Award*
- 2014 Visiting researcher for BIOL 307, Carleton College’s semester abroad in NZ

Training activities

- 2011 International Workshop in Evolutionary Biology. Univ. of Basel. Guarda, Switzerland.
- 2009 Evolutionary Biology Workshop in the Alps. Univ. of Lausanne. La Fouly, Switzerland.
- 2009 Third FEBS Advanced Lecture Course: Human Fungal Pathogens. Nice, France.

Professional Service

- Reviewer Nature Ecology and Evolution; Evolution; the American Naturalist; Scientific Reports; Infection, Genetics and Evolution; BMC Evolutionary Biology; Agricultural Science Research; PLoS One
- Societies European Society for Evolutionary Biology; Society for the Study of Evolution; the American Society of Naturalists; Indiana Academy of Science; Sigma Xi

Community Outreach

- 2011- present Monthly volunteer for Science of Art Evening, Wonderlab Museum, IN
- 2011- present Annual volunteer at 4th Street Festival and Wonderlab Art Booth, IN
- 2015 Volunteer and moderator for Midwest Ecology and Evolution Conference, IU, IN
- 2014 Biology volunteer at IU Science Fest, Passenger Pigeon Origami Project, IN

2013	Summer research mentor for a high school junior, Matthew Johnsen, IU, IN
2012-2013	Science Olympiad coach, Disease Detectives event, Bloomington High School South, IN
2011-2013	Mentor of four semester-long independent research projects, Harmony High School, IN
2009-2010	Mentor of year-long independent research project, Woodrow Wilson High School, DC; <i>*Suliman Abdullah received honors at the DC Environmentors Science Fair</i>